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Growth of escherichia coli by oxygen absorption rate controlled fed batch cultivation

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GROWTH OF ESHERICHIA COLI BY OXYGEN ABSORPTION RATE CONTROLLED FED BATCH CULTIVATION

by

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CERTIFICATE OF APPROVAL

This research report is accepted and approved in partial fulfillment of the requirements for the degree of Master of Science in Chemical Engineering.

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27 May 1982
(date)

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Chairman
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To my family

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I would like to thank Dr. Marvin Charles for his support, interest and witty advice during the course of this work. Thanks also to everyone in the department for they made my stay here a most enjoyable and rewarding experience.

I am especially gratefull to my parents and uncle Achilles for their encouragement, patience and financial support without which the completion of this work would have been impossible.

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ABSTRACT

The use of dissolved oxygen concentration measurements to regulate the substrate feed to a fed batch fermentation for the production of Esherichia coli has been studied and discussed.

Experiments were conducted in a conventional 7 liter bench scale stirred tank and a 300 liter pilot scale deep jet aeration fermenters. A simple on-off control system was used and the substrate was glucose.

The controller was functional and cell mass concentrations up to 40 g/l were achieved. The experimental results were in good agreement with the dynamic behavior predicted by a computer simulation of the process.

1. INTRODUCTION

The growth of microorganisms to high concentrations with low power requirements and efficient nutrient utilization has always been a subject of tremendous interest in fermentation technology.

In a batch fermentation system, where a microorganism is grown on a balanced medium which contains enough substrate for unrestricted growth, the factor which limits the cell concentration to about 10 g dry cells/l[1] is the oxygen supply rate to the culture. Furthermore, Pirt[2] and Nilson[3] have found that the growth yield with respect to the substrate is considerably lower in such batch cultures than in cultures where the rate of growth is restricted by the concentration of the carbon source, an effect that remains unexplained.[4] Therefore, growth of microbes to high concentrations with high substrate yield coefficients depends on the ability of the fermentation system to ensure oxygen availability and substrate restricted growth.

By keeping the oxygen demand of the culture below the maximum oxygen transfer rate (MOTR) of the fermenter, Bauer[5] was able to grow E coli on sucrose to about 42 g dry cells/l. This was achieved by regulating the substrate feed so as to limit the microbial growth rate, thereby keeping the oxygen demand below the fermenter's MOTR. The substrate feed flow rate was regulated according to the level of the dissolved oxygen concentration in the culture. Such oxygen absorption controlled fed batch fermentation has been used in

industry[4,6] and was analyzed first by Hospodka[7] who derived the fundamental equations that describe the system.

Fed batch cultivation has a number of other advantages. It can be used to overcome the inhibitory effects of precursors (e.g. phenylacetic acid) and other medium components. Furthermore, repeated fed batch cultivation, where the continuous increase of the total culture volume is interrupted by removing periodically a portion of the culture can provide productivity greater than that achieved by simple repeated batch fermentation and, in some cases, approaching that attainable by continuous fermentation. The penicillin fermentation is now run routinely as a repeated fed batch, as are baker's yeast fermentations and waste treatment processes. The theory of fed batch cultivation has been the subject of several publications[4,8], and computer simulations have been developed.[6,8]

In this work E coli was grown to high concentrations under dissolved oxygen controlled fed batch cultivation. The substrate was glucose. A simple control system was used to regulate the glucose flow into the fermenter according to the dissolved oxygen concentration level. The resulting low glucose concentration in the culture ensured substrate limited growth and the oxygen demand was kept below the MOTR by maintaining constant dissolved oxygen concentration.

Experiments were carried out in a conventional stirred tank bench scale fermenter and in a 300 l deep jet aeration fermenter

(recirculation fermenter). The Vogelbush IZ deep jet aeration fermenter that was used in the experiments is illustrated in Figure 1. According to the concept of its operation, oxygen transfer and agitation are accomplished by using a high velocity recirculation loop. The culture broth is recirculated by means of a specially designed compound pump (Figure 2) consisting of an Archemedes screw booster followed by a vented centrifugal section. The design and construction of the impeller and volute chamber make the pump able to handle liquid-gas mixtures with density as low as 0.6 g/ml. The recirculating broth aspires sterile air as it flows over a circular weir, thereby creating a venturi effect. Air is aspired at rates higher than 600 slpm, thus making the use of an air compressor necessary only when the system is operated at pressures higher than atmospheric. The vertical return leg of the recirculation loop acts as a wetted wall column and the high velocity liquid jet that is formed by the returning broth provides intense plunging - jet mixing for improved oxygen transfer with low power requirements. Heat transfer occurs as the broth is pumped through the jacketed loop, independent of the fermentation tank, thereby providing two distinct zones for mass and heat transfer.

In addition to the excellent heat and mass transfer characteristics and its low power requirements, the deep jet aeration fermenter is relatively simple to scale up and therefore, it should be considered as an alternative to stirred tank fermenters for research and industrial use.

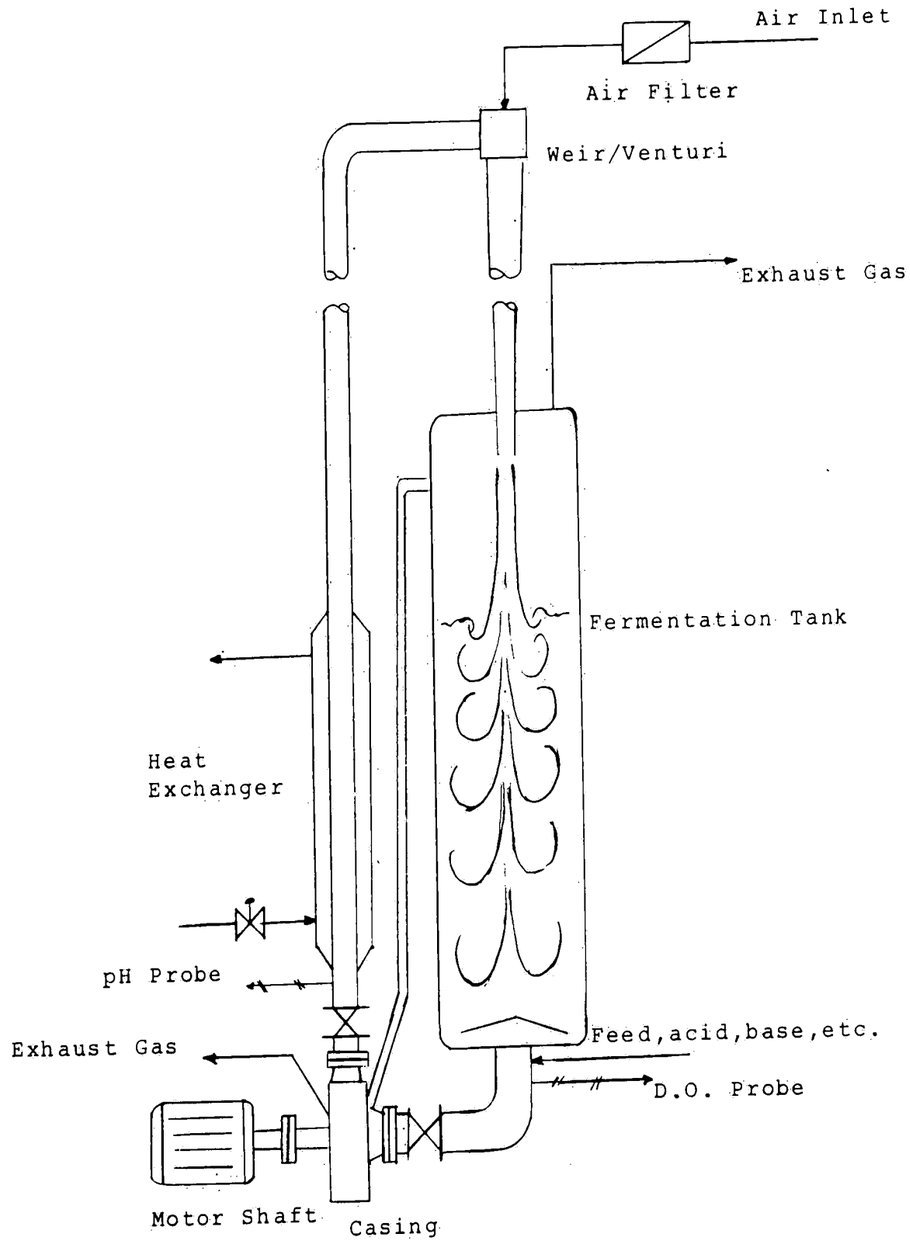
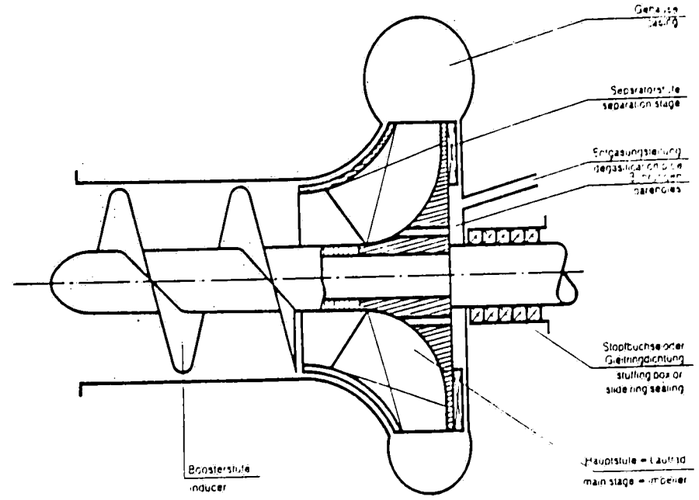
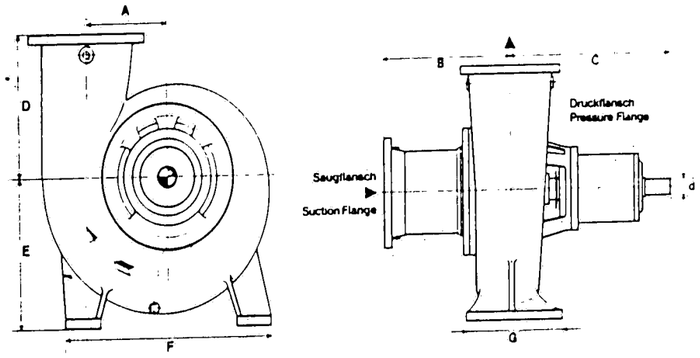


Figure 1: The Deep Jet Aeration Fermenter





Größe	Saugflansch mm	Druckflansch mm	A	B	C	D	E	F	G	d
10 MN - IZ	250	250	230	430	730	340	350	600	300	60
12 MN - IZ	300	300	280	500	835	355	400	700	350	70
16 MN - IZ	400	400	370	700	1010	470	530	850	480	80
20 MN - IZ	500	500	460	850	1050	740	700	1050	600	100
24 MN - IZ	600	600	560	1000	1100	710	810	1250	750	100
30 MN - IZ	800	800	560	1000	1320	820	1050	1540	800	108

Figure 2: Cross Sectional Schematic and Main Dimensions of the Vogelbush IZ Compound Pump

The primary objectives of the experiments were to evaluate the effectiveness of the oxygen control system and to investigate and compare the overall performance of the process in the bench-scale stirred tank and pilot-scale deep jet aeration fermenters.

A computer simulation of the process was developed using the model which results from the equations that describe this fed batch system. A parametric study was then performed on the simulation in order to determine the dynamic behavior of the process for different values of quantities such as the oxygen transfer coefficient and the Monod's kinetic parameters.

2. THEORY

In a fed batch cultivation the total mass balance statement is:

$$\left[\begin{array}{c}\text{Rate of mass accumulation}\\ \text{within the fermenter}\end{array}\right] = \left[\begin{array}{c}\text{Rate of mass flow}\\ \text{into the fermenter}\end{array}\right]$$

Assuming that all nutrients are added in one stream and neglecting any mass losses due to ammonia evaporation, carbon dioxide emission and moisture in the exhaust air, the above statement becomes:

$$\frac{d(V\rho)}{dt} = F \tag{2.1}$$

where

V : total volume of liquid in the fermenter l

ρ : liquid density g/l

t : time hr

F : total mass flow rate into the fermenter g/hr

Expanding the differential and assuming constant density system, the mass balance becomes:

$$\frac{dV}{dt} = \frac{F}{\rho} \tag{2.2}$$

Notice that F can be a function of time.

Under the same considerations, the mass balance based on the biomass concentration may be written as:

$$\left[\begin{array}{c}\text{Rate of biomass accumulation} \\ \text{within the fermenter}\end{array}\right] = \left[\begin{array}{c}\text{Rate of biomass} \\ \text{generation}\end{array}\right]$$

Therefore, for a well mixed culture broth:

$$\frac{d(Vx)}{dt} = \mu x V \tag{2.3}$$

where

x : biomass concentration g/l

μ : specific growth rate hr^{-1}

The specific growth rate is assumed to be dependent on the substrate concentration according to the Monod[9] kinetic expression:

$$\mu = \frac{\mu_{max} S}{(K_s+S)} \tag{2.4}$$

where

μ_{max} : maximum growth rate constant hr^{-1}

K_s : saturation constant g/l

S : substrate concentration g/l

Generally, Monod's equation applies for cultures under steady state or slowly changing conditions[8], where balanced growth occurs. Therefore, if it is applied to fermentation systems where the culture is subject to sudden changes in pH, temperature or nutrient concentration the results can be erroneous. Other more complex kinetic models have been proposed, but Monod's expression is accurate for E coli for

constant temperature and pH and midly varying dissolved oxygen and substrate concentrations. It is used here for its simplicity.

If μ and dV/dt are substituted in the expanded form of Equation 3 via Equations 1 and 4 respectively, the biomass balance becomes:

$$\frac{dx}{dt} = \left(\frac{\mu_{max} S}{(K_s+S)} - \frac{F}{\rho V}\right) x \qquad (2.5)$$

The substrate mass balance is:

$$\begin{bmatrix}\text{Rate of substrate} \\ \text{accumulation}\end{bmatrix} = \begin{bmatrix}\text{Rate of substrate} \\ \text{flow} \\ \text{into the fermenter}\end{bmatrix} - \begin{bmatrix}\text{Rate of} \\ \text{substrate utilization}\end{bmatrix}$$

That is:

$$\frac{d(SV)}{dt} = Fy - \frac{\mu x V}{Y_G} - \frac{\alpha_G x V}{Y_G} \qquad (2.6)$$

where

y : weight percent concentration of glucose in the feed stream

α_G : glucose specific maintenance coefficient hr^{-1}

Y_G : glucose yield coefficient g cells dry wt/g glucose

To simplify the model, the specific maintenance and yield coefficients are assumed to be constant; however, it should be recognized that there is conflicting evidence concerning the validity of this assumption for the system under study. Expanding the differential and using Equations 2 and 4, as with the biomass balance, the substrate balance becomes:

$$\frac{dS}{dt} = \frac{F}{V}(y - \frac{S}{\rho}) - [\frac{\mu_{max} S}{(K_s+S)} + \alpha_G] \frac{x}{Y_G} \qquad (2.7)$$

Equations 2, 5 and 7 constitute a system of three equations in S, x and V that model a simple fed batch fermentation process. In oxygen controlled fed batch the substrate flow rate, Fy, is the variable that is manipulated to control the dissolved oxygen concentration in the broth. The flow rate, F, is not constant with time hence, two additional equations in F and the dissolved oxygen concentration, C, are needed. The first equation is supplied by an oxygen balance:

$$\begin{bmatrix}\text{Rate of oxygen} \\ \text{accumulation}\end{bmatrix} = \begin{bmatrix}\text{Rate of oxygen} \\ \text{absorption}\end{bmatrix} - \begin{bmatrix}\text{Rate of oxygen uptake} \\ \text{by the organism}\end{bmatrix}$$

The oxygen absorption term is usually expressed as:

$$OTR = Kla\ (C^* - C) \qquad (2.8)$$

where

OTR : oxygen transfer rate g/l-hr

Kla : overall oxygen mass transfer coefficient hr^{-1}

C^* : equilibrium dissolved oxygen concentration g/l

C : bulk dissolved oxygen concentration g/l

Since oxygen transfer occurs over an integral volume a mean driving force, $(C^* - C)_{\text{volumetric mean}}$, should be used in Equation 8. However, in a well mixed reactor C can be assumed uniform throughout the volume.

Also, Tagucki and Humphrey[10] have shown that C^* changes only slightly during the course of most fermentations. Therefore, a simple difference is a reasonable approximation of the true driving force. Taking this approach, the dissolved oxygen balance becomes:

$$\frac{d(CV)}{dt} = Kla(C^* - C)\ V - QxV - \frac{\alpha_{O_2} xV}{Y_{O_2}} \tag{2.9}$$

where

Q : specific oxygen uptake rate g_{O_2}/gcells-hr

α_{O_2} : specific oxygen maintenance coefficient hr^{-1}

Y_{O_2} : oxygen yield coefficient g cells/g_{O_2}

The specific oxygen uptake rate can be based on the specific growth rate and therefore, on the substrate concentration via Monod's equation as follows:

$$Q = \frac{\mu}{Y_{O_2}} = \left[\frac{\mu_{max} S}{(K_s+S)}\right] \frac{1}{Y_{O_2}} \tag{2.10}$$

After expanding the differential, substituting for dV/dt via Equation 2 and Q via Equation 10 and assuming constant coefficients, Equation 9 develops to:

$$\frac{dC}{dt} = Kla(C^* - C) - \frac{FC}{V\rho} - \left[\frac{\mu_{max} S}{(K_s+S)} + \alpha_{O_2}\right] \frac{x}{Y_{O_2}} \tag{2.11}$$

The second equation is the one that describes the action of the controller and has the form identified with the type of control used. On-off

pulse control (Section 3.4.2) was used in these experiments. The action of pure on-off controller can be approximated best by the equation that describes a high gain proportional controller[11]:

$$F = Fo + KC\ (C - C_{sp}) \tag{2.12}$$

where

Fo : steady state value (= 0 for on-off control) g/hr

KC : controller gain (high value for on-off control).

C : dissolved oxygen concentration g/l

C_{sp} : set point dissolved oxygen concentration g/l

An important part of every control system is the device (transmitter) that detects the value of the controlled parameter and translates it to a signal to be received by the controller. For the oxygen control outlined here, the transmitter used was a galvanic membrane covered oxygen electrode. Its dynamic response to a change in dissolved oxygen concentration can be represented by the first order lag model[12]

$$\frac{dC_E}{dt} = \frac{C - C_E}{\tau_{EF}} \tag{2.13}$$

where

C_E : dissolved oxygen concentration as detected by the electrode g/l

C : true dissolved oxygen concentration g/l

τ_{EF} : time constant referring to the diffusion film and the electrode (only for nonviscous liquids) hr.

The effect of mixing lags is ignored here since perfect mixing was assumed earlier to simplify the model.

Equations 2, 5, 7, 11, 12 and 13 provide the model of a fed batch microbial growth under dissolved oxygen controlled substrate feeding. Pirt[4] has analyzed the dynamic behavior of a simple fed batch model (Equations 2,5 and 7) and Dunn and Mor[8] have compared it to the constant volume chemostat. They assumed constant feed flow and they showed that "a dynamic steady state is achieved for sufficiently low flow rates such that the specific growth rate is exactly maintained equal to the dilution rate", or $F/V\rho = \mu$. Equation 5 then suggests that $dx/dt = 0$. In this respect fed batch behaves just like the chemostat. However, since the volume increases steadily, the substrate concentration must decrease to maintain equality between $F/\rho V$ and μ (Eq. 5). This situation is not a true steady state since only x is constant. The term "quasi steady state" is used, after Pirt, to describe the phenomenon. The model of the oxygen controlled fed batch should behave analogously since the variable flow rate is the only difference. These aspects will be discussed in some length later.

At this point the assumptions embodied in the foregoing development should be summarized:

a) No mass losses (flow into the fermenter only):

b) constant temperature, pH, density, yield and maintenance coefficients;

c) single substrate limitation;

d) balanced growth;

e) Monod kinetics;

f) (C^* - C) driving force for oxygen transfer;

g) constant oxygen transfer coefficient; and

h) perfect mixing.

3. EXPERIMENTAL

3.1 Organism

Escherichia coli strain W-3100 (ATCC E 19498) was used in all experiments.

3.2 Medium

The medium used for the fermentations and the starter cultures had the following composition (g/l), after Bauer[13]:

K_2HPO_4	3.0
KH_2PO_4	3.0
$(NH_4)_2HPO_4$	1.0
$MgSO_4 \cdot 7H_2O$	2.0
anhydrous glucose grade III	5.0
yeast extract (BBL)	5.0

A solution of trace elements was added to the medium to a concentration of 3 ml of trace elements solution per liter of medium. The trace elements solution had the following composition[13] (g/l):

$FeCl_3 \cdot 6H_2O$	27.0
$ZnCl_2 \cdot 4H_2O$	2.0
$CoCl_2 \cdot 6H_2O$	2.0
$NaMoO_4 \cdot 2H_2O$	2.0
$CaCl_2 \cdot 2H_2O$	1.0
H_3BO_3	0.5
HCl (conc.)	100 ml

A solid persipitate, presumably a complex magnesium - potassium phosphate, appeared after sterilization of the medium. Since its presence caused no obvious adverse effects it was ignored. After sterilization, the pH of the medium was 6.6. Shake flasks were sterilized containing the complete medium; however, all fermenters were sterilized without glucose to prevent its caramelization by the phosphate reaction. Glucose was sterilized separately and then added to the medium to the desired concentration before inoculation.

3.3 Seed and Starter Cultures

3.3.1 Seed preparation.

The seed cultures were prepared as follows:

a) A stock culture was transferred to nutrient broth agar slants, which were incubated at 35 °C for thirty two hours.

b) From the agar slants the cultures were transferred to 250 ml shake flasks containing 50 ml of sterile medium. The cultures were grown at 35 °C and 250 rpm in a New Brunswick-G-26 incubator shaker for 16 hours.

c) The 50 ml cultures then were transferred to 2 liter shake flasks with 450 ml of medium. As before, the cultures were grown at 35 °C and 250 rpm for 16 hours, thus giving cultures at their late logarithmic phases[11].

3.3.2 Starter Cultures

The 2 liter shake flasks containing 500 ml of culture were used directly as starter cultures to inoculate the 7 l bench scale fermenters.

A 14 l New Brunswick Model 19 fermenter, with 10 l of medium was used to inoculate the deep jet aeration fermenter. It was sterilized at 121 °C for 1 hr, inoculated with 500 ml of seed culture and operated at 35 °C and 400 rpm to give a starter culture containing about 6 g dry cells/l. Before inoculation, a concentrated glucose solution was sterilized separately and added to the fermenter to a concentration of about 20 g glucose per liter of medium.

3.4 Fermentations and Dissolved Oxygen Control

3.4.1 Bench Scale

The bench scale experiments were carried out in a 7.5 l New Brunswick MF-107 fermenter initially containing 3 l of medium. Agitation was provided by two pitched blade impellers operated at 600 rpm. Air was sparged underneath the lower impeller at a rate of 6 lpm. The pressure was atmospheric. Foam was controlled by automatic addition of General Electric AF72 Silicon Antifoam (50% $^{v}/v$). The temperature was maintained at 35 °C, and the pH at 6.8. The pH control system operated as follows: An Ingold steam sterilizable pH probe connected to a New Brunswick pH-22 controller activated a Masterflex No 7014 peristaltic pump, which in turn introduced small amounts of 18.7% ($^{w}/w$) ammonia solution into the fermenter whenever the pH dropped below the set point. The ammonia was assumed to serve adequately as nitrogen source and therefore, no other nitrogen was added except for the nitrogen initially present in the medium. The ammonia consumption was determined by the difference in the weight of the addition vessel. A solution of K_2HPO_4, KH_2PO_4 and $MgSO_4 \cdot 7H_2O$ was added periodically to the culture to

prevent interruption of the growth due to exhaustion of the initially introduced potassium, phosphorous and magnesium. The amount of salts added was determined from cell concentration estimations and the elemental composition of E coli[14].

3.4.2 Dissolved Oxygen Control

The dissolved oxygen control system is illustrated in Figure 3. A galvanic steam sterilizable dissolved oxygen probe[15] (Abec series A316) was connected to a New Brunswick DO-50 dissolved oxygen analyzer - recorder. The 0 - 10 mV signal from the analyzer was transmitted to a Speedomax H Leeds and Northrup controller which in turn was connected to a timer. The timer (Atc Series 365 Computing Timer) was programmed to generate a pulse output of adjustable duration each time it received signal from the controller, i.e., each time the dissolved oxygen concentration rose above the set point. The timer output activated an adjustable flow rate Masterflex No 7016 peristaltic pump which pumped 58% ($^{w}/w$) glucose solution into the fermenters. The set point dissolved oxygen concentration was 40% of the saturation value.

The control system was activated the first time the dissolved oxygen concentration dropped below the set point. Enough glucose was supplied before inoculation to support growth up to this point only. As with ammonia, the glucose consumption was recorded.

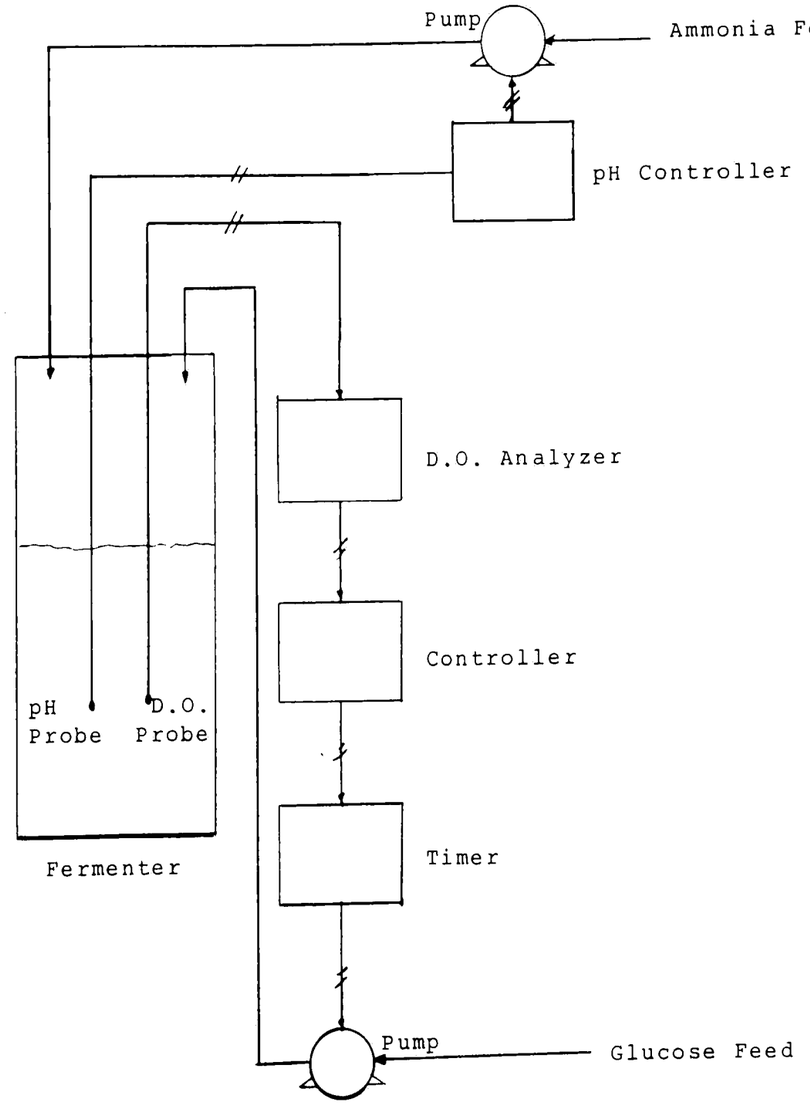


Figure 3: Dissolved Oxygen and pH Control

3.4.3 The Deep Jet Aeration Fermenter

3.4.3.1 Fermentation System

The Vogelbush IZ deep jet aeration fermenter used for the experiments was described in the Introduction (Section 1.) A temperature PID controller, A pH proportional pulse controller, a PID dissolved oxygen controller, which adjusts the pump's speed and automatic foam control are built into the system. Furthermore, four 25 l stream sterilizable agitated tanks are connected to the fermenter and are used in conjunction with the controllers for acid, base, nutrient and antifoam additions.

3.4.3.2 Operation

The deep jet aeration fermenter was operated with the oxygen controller described in Section 3.4.2. The procedure was as with the bench scale experiments (Section 3.4.1). The operating conditions were as follows:

Temperature	:	PID control, T = 35 °C
Pressure	:	Approximately 1.2 atm
pH control	:	Proportional pulse, pH = 6.8, 12% $^{w}/_{w}$ ammonia solution
Pump speed	:	900-920 rpm; control on manual
Initial volume	:	200 l
Recirculation flow rate	:	Approximately 600 lpm
Air flow rate	:	650-700 slpm

Dissolved oxygen set point concentration : 40% saturation

Glucose feed : 58% ($^{w}/w$)

Antifoam : 10% ($^{v}/v$) G.E. AF 72 silicon antifoam

Ammonia, salts and antifoam were added from the tanks mentioned in Section 3.4.3.1. Glucose was added from separate glass vessels:

a) To allow direct weighing and

b) because the dissolved oxygen control system could not be interfaced easily with the deep jet's addition tanks.

3.5 Analytical Methods

Samples of the culture were taken every 1.5 hours. Glucose concentration was determined by means of a YSI Model 23A glucose analyzer. Biomass concentration was determined as follows:

a) Vacuum filter 3 ml of culture broth through 0.45 micron pore diameter membranes;

b) wash the filtrate with 5 ml of deionized water;

c) dry cell-laden membranes at 105 ℃ overnight;

d) measure the weight of the dry cell-laden membranes and calculate the dry cell weight by subtracting the predetermined weight of the clean-dry membranes.

Figure 4 shows a cell mass concentration versus turbidity correlation which was derived experimentally and used only to estimate the cell mass concentration during the fermentations. The turbidity of the samples, diluted to 1.7% ($^{v}/v$) with deionized water, was determined by means of a Baush and Lomb Spectronic 700 spectrophotometer at 540 nm.

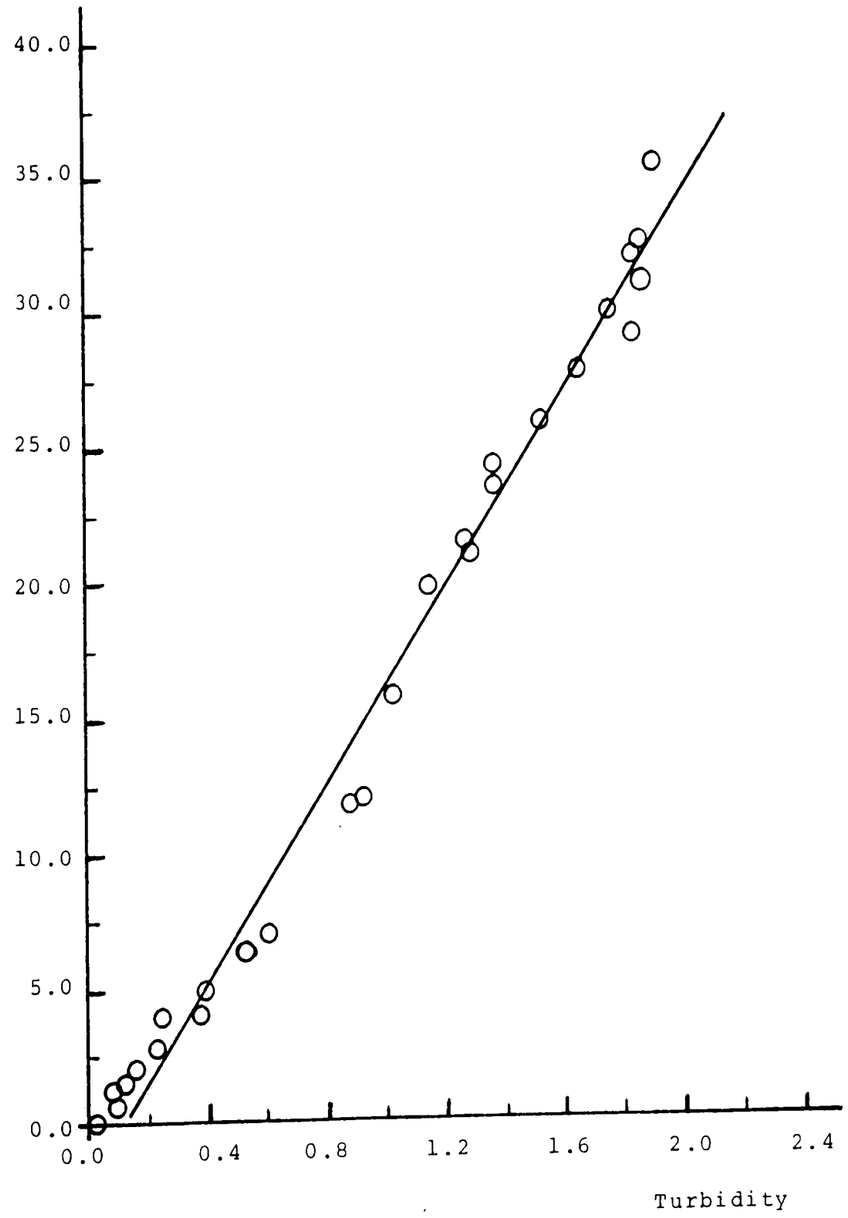


Figure 4: Turbidity Versus Cell Mass Concentration, 1.7% ($^{v}/v$) Dilution of Samples, 540 nm.

4. COMPUTER SIMULATION

The process was simulated using the mathematical model defined by Equations 2.2, 2.5, 2.7, 2.11 and 2.12. The dynamic behavior of the model was obtained by simultaneously solving the above equations via the LSODE initial value ordinary differential equation solver routine[16], written in FORTRAN for a digital computer. It should be noted that the results of the simulation can only be a first approximation of the dynamic behavior of the actual process, considering the assumptions and simplifications embodied in the development of the model (Section 2).

For the simulation, the parameters of the model that characterize E coli were chosen from the literature. Other process parameters were chosen to fit experimental data.

The values of the glucose and oxygen yields and their respective maintenance coefficients, as well as their dependence on environmental conditions is a subject of considerable controversy. The values reported by Mainzer and Hampfling[17] in their study of the effects of temperature on the oxygen yield and maintenance coefficients were used in this simulation. At 35 °C the yield and maintenance coefficients for glucose limited growth of E coli B were found to be approximately 14 g dry cells/g atom O and 2 mg atom O/g dry cells-hr respectively; the temperature dependence of the coefficients was, unexplainably, rather eratic. Subject to similar uncertainty are the values of 0.5 g dry cells/g glucose and 0.028 hr^{-1} for glucose yield and maintenance coefficients respectively, that were used here as reported by Marr et al[18] for E coli PS.

The equilibrium oxygen concentration at 35 °C was assumed to be equal to 7.4 ppm, in agreement with values reported[10,19,20] for solutions similar to the medium used in the experiments. The set point concentration was set to 40% of this value.

The generally accepted value for the saturation constant in the Monod expression (Eq. 2.4) is 2.2×10^{-5} M, or about 4.0 mg/l for E coli grown or glucose[21,22,23,24]. Wang et al[24] report a growth rate of 0.82 hr^{-1} for unrestricted growth of E coli on glucose and Shuler[23] gives a μ_{max} of 0.95 hr^{-1}. To investigate the effects of the Monod's constants on the dynamic behavior of the model, the computer program was run for three different sets of Monod's constants all other parameters being held constant. Similarly, the program was run for four different oxygen transfer coefficients, all other parameters being constant. Table 1 summarizes the parametric study.

To simplify the programming, glucose, ammonia, antifoam and salt additions were considered one feed stream containg 36% ($^{W}/w$) glucose. The above figure for the glucose concentration in the feed was calculated from experimental data on additions by dividing the total weight of the glucose added during the fermentation by the total weight of all additions.

The equation of a high gain proportional controller was used in the simulation to approximate the type of "pulse" on-off controller used in the experiments (Section 3.4.2). The responses of the manipulated variable, F, to changes in the control variable, C, for proportional, pure on-off and pulse on-off control are shown graphically in Figure 5.

Table 1: Parametric Study

Run #	K_s(g/l)	μ_{max}(hr^{-1})	Kla(hr^{-1})
1	0.004	0.95	300.0
2	0.004	0.95	500.0
3	0.005	0.75	500.0
4	0.006	0.55	500.0
5	0.004	0.95	700.0
6	0.004	0.95	900.0

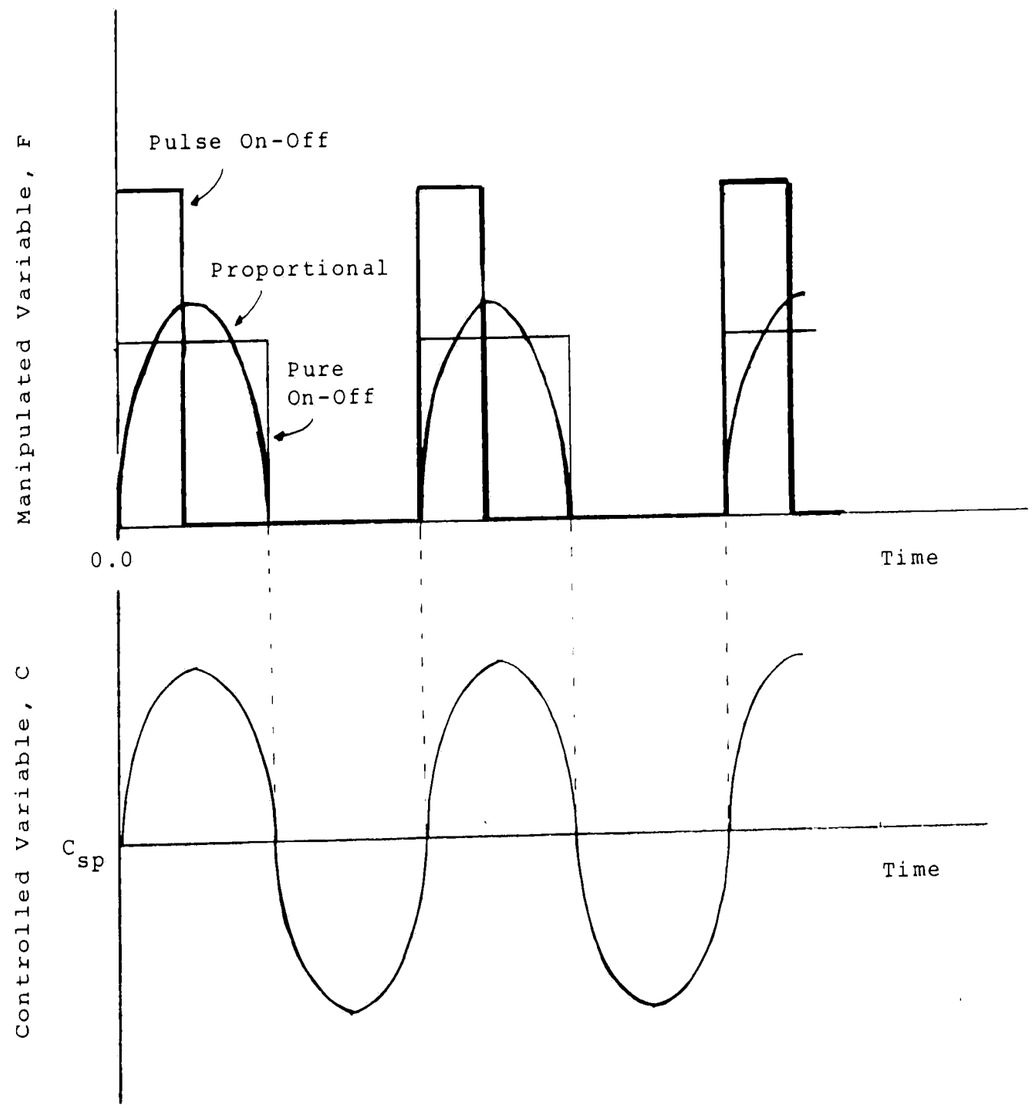


Figure 5: Responses of Pulse on-off, Pure on-off and Proportional Control to Changes in the Controlled Variable.

The program was organized in such a manner as to ignore the action of the controller when the dissolved oxygen concentration was lower than the set point value. If no such action was taken, the program would generate negative values since the steady state flow rate (Eq. 2.12) was zero.

The equation that represents the measurement lag (Eq. 2.13) was not incorporated in the simulation. Similarly, the effects of the mixing lag were ignored since the purpose of this modeling was to give an insight into the performance of fed batch cultivation rather than to investigate the function of different types of control. Proportional control was used to facilitate the programming. The computer simulation of the action of an on-off controller is relatively simple. However, the discontinuities (see Figure 5) in the time behavior of the manipulated variable, that are inherent in any type of on-off control, made its use inefficient in terms of computer time, because a large number of progressively smaller steps was required by the integrator to pass through each discontinuity and carry out the simulation. The use of proportional control eliminated such discontinuities and, as it turned out, the dissolved oxygen concentration was maintained constant during the simulation and the only difference between proportional and on-off control (lags excluded from the simulation) was the small offset error (app. 0.1%), which is a characteristic of proportional control[11]. The savings in computer time though, were remarkable.

A complete run (No. 4 in Table 1) of the computer program is given in the Appendix.

5. RESULTS AND DISCUSSION

The growth curves generated by the computer simulation for different oxygen transfer coefficients, runs 1, 2, 5 and 6 in Table 4.1 are plotted in Figure 6. As theory predicts, the biomass concentration reaches a plateau and the specific growth rate decreases (Figure 7) as the quasi steady state is approached (Eq. 2.5). Consequently the glucose concentration in the culture also decreases (Figure 8) due to the relationship of the specific growth rate to the substrate concentration as expressed by Monod's equation (Eq. 2.4). The above mentioned figures show that high Kla values, i.e., high rates of oxygen transfer to the culture result in high biomass concentrations and high specific growth rates at the quasi steady state.

The simulation was not carried out to include the quasi steady state because stability studies and hence, tuning of the control system were not an objective of this work. As it will be discussed later, the computer simulation can be implemented to facilitate verification of the existence of the quasi steady state as predicted, and study of the performance of the process at the quasi steady state.

Equations 2.4 and 2.10 state the dependence, according to Monod's expression, of the specific growth rate and specific oxygen demand on the substrate concentration. It is this dependence of the specific oxygen demand on the substrate concentration that constitutes the basis of dissolved oxygen control by manipulating the rate of glucose flow into the culture. These relationships are the subject of Figure 9, which

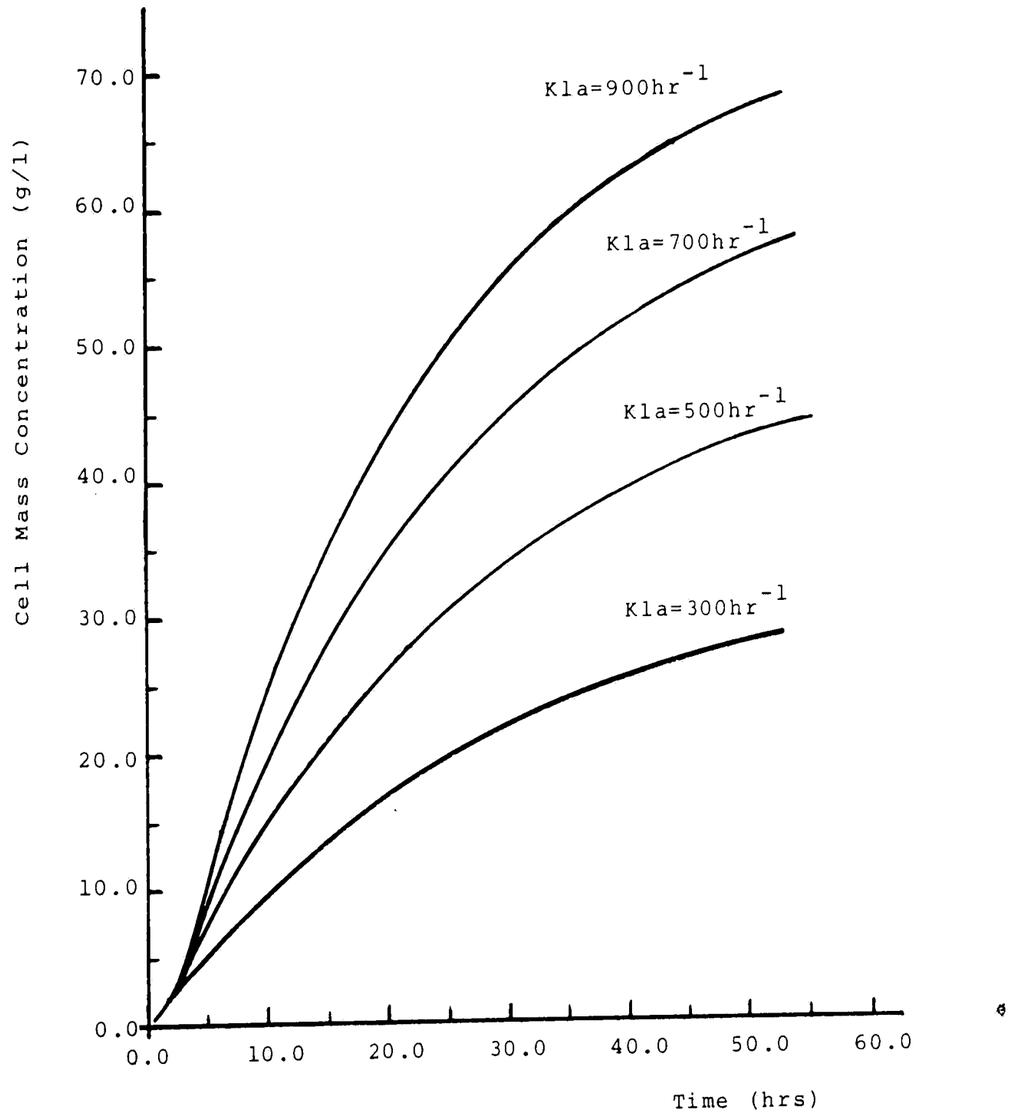


Figure 6: Cell Mass Concentration Versus Time for Various Oxygen Transfer Coefficients; Computer Simulation, K_s = 0.004 g/l, μ_{max} = 0.95 hr^{-1}.

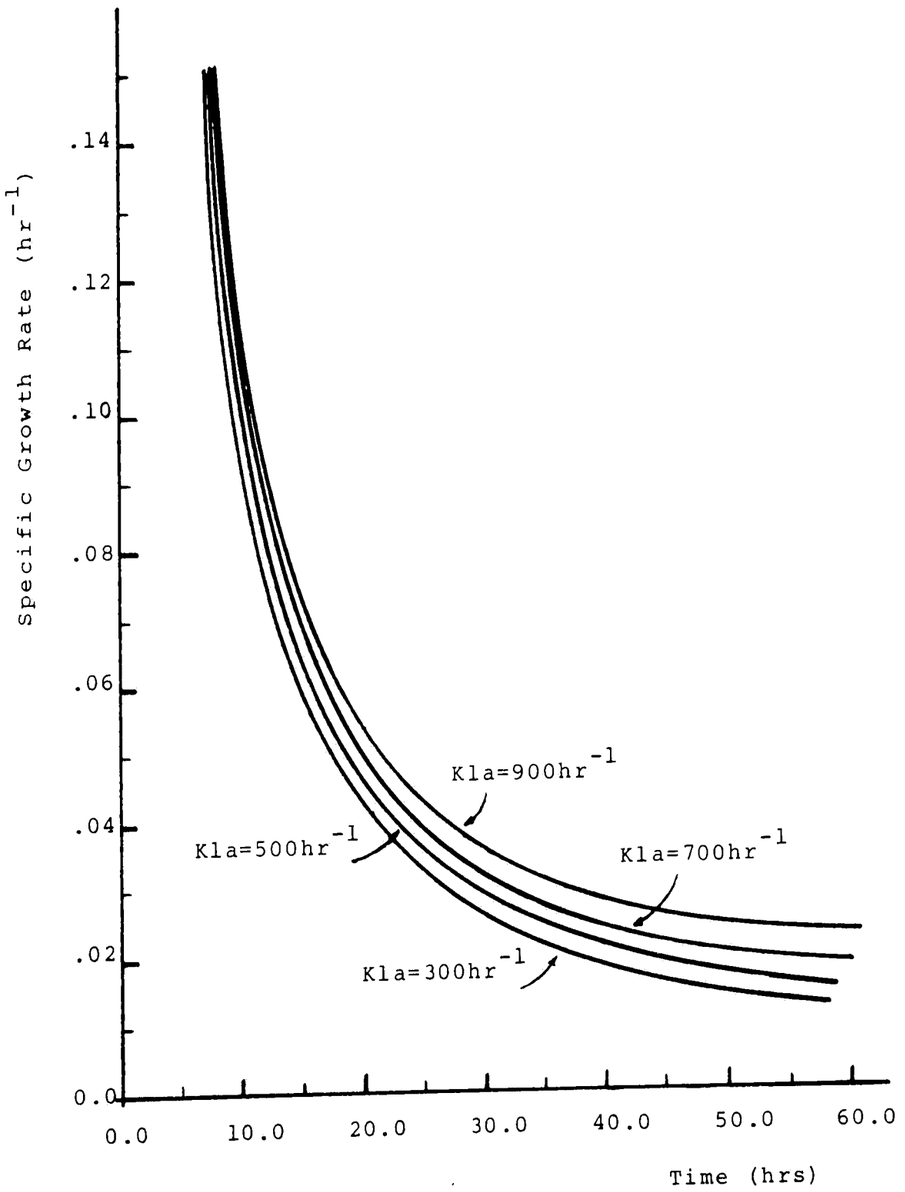


Figure 7: Specific Growth Rate Versus Time for Various Oxygen Transfer Coefficients; Computer Simulation, K_s = 0.004 g/l, μ_{max} = 0.95 hr^{-1}.

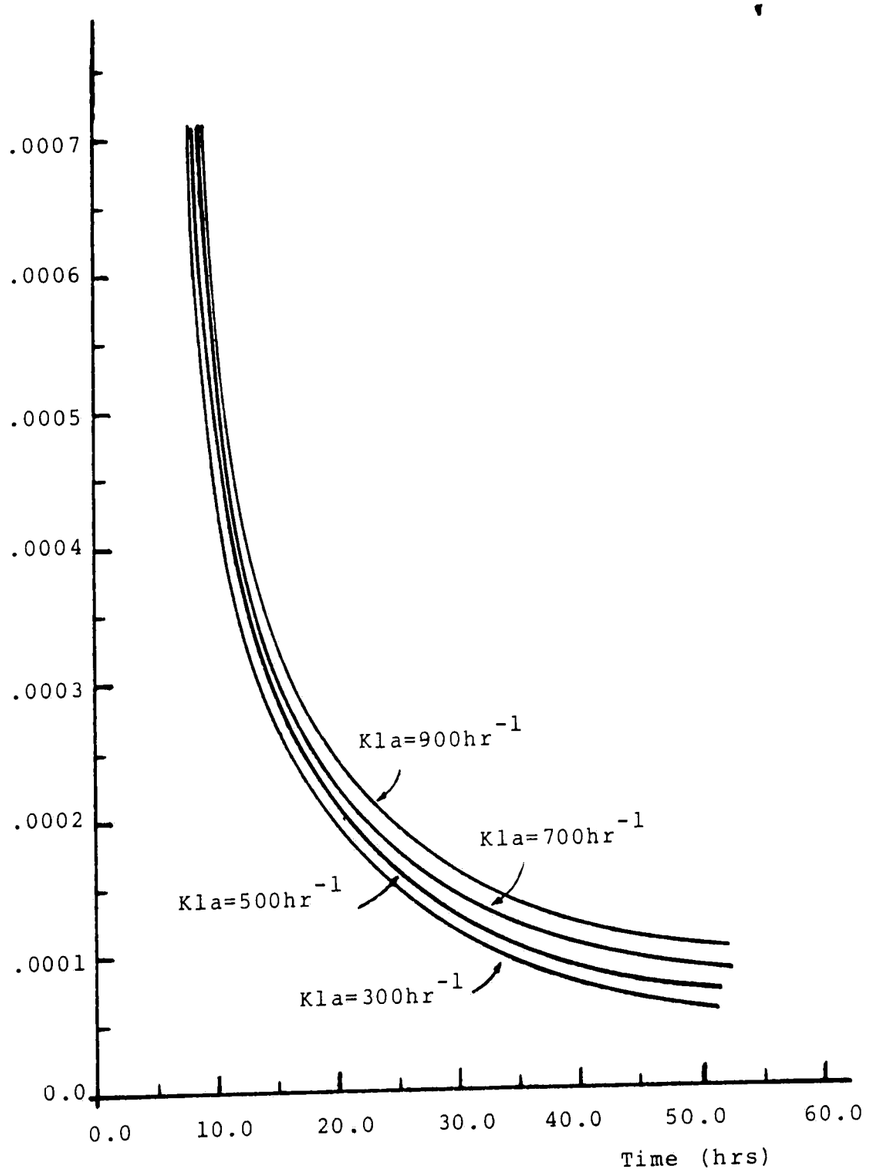


Figure 8: Glucose Concentration Versus Time for Various Oxygen Transfer Coefficients; Computer Simulation, K_s = 0.004 g/l, μ_{max} = 0.95 hr^{-1}.

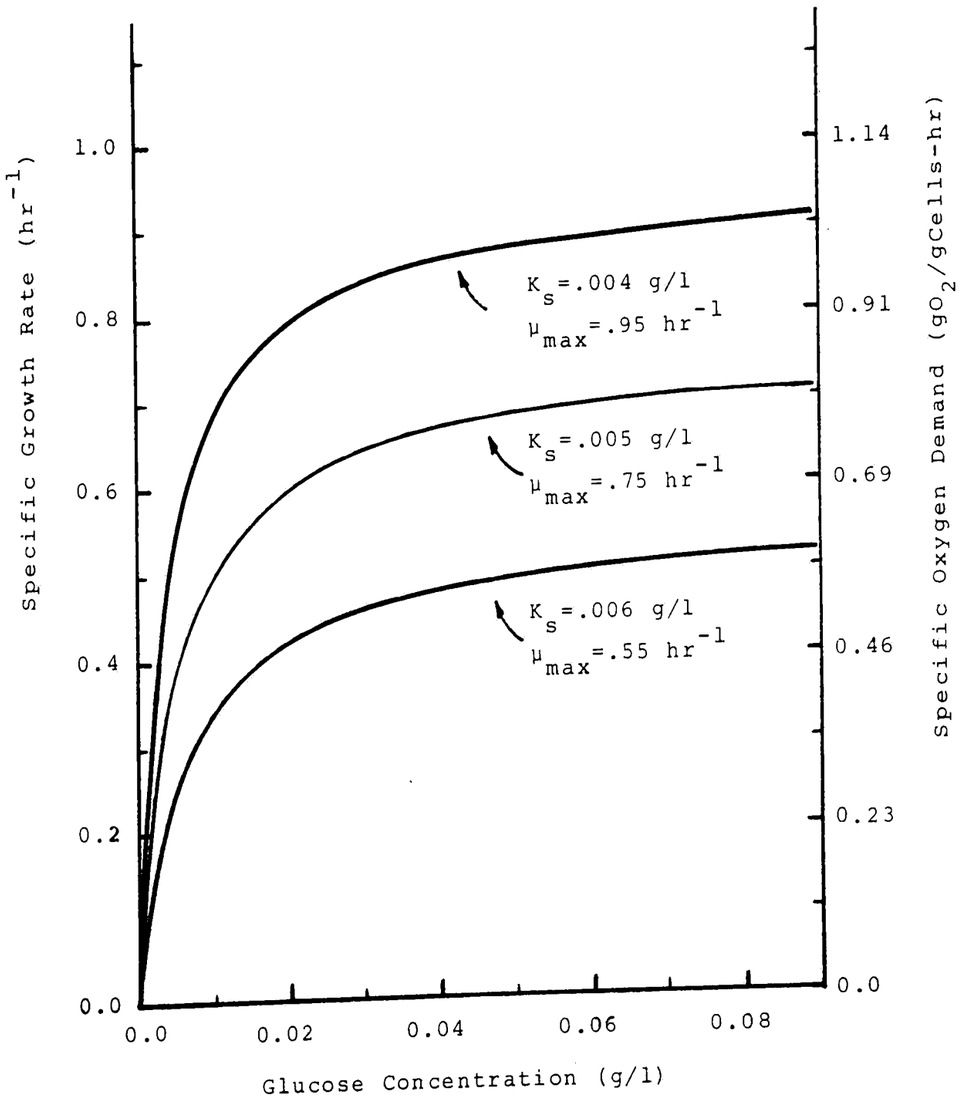


Figure 9: Specific Oxygen Demand and Specific Growth Rate Versus Glucose Concentration. (Monod Kinetics, Y_{O_2} = 0.875)

shows that the specific oxygen demand is quite sensitive to very small changes in the glucose concentration in an E coli culture, especially for concentrations lower than 0.01 g/l. The high slope of the glucose concentration versus specific growth rate (and oxygen demand) curves and the rather low concentrations (less than 0.0003 g/l; see Figure 8) at which control is feasible, are the result of the low values of the saturation constant, K_s, that characterize the glucose - E coli system. Gleiser and Bauer[5] have shown for example, that E coli grown on sucrose exhibits a K_s of 10 g/l which makes the fermentation controllable over a wider range of higher substrate concentrations. However, for glucose, the low K_s value in conjunction with the measurement and mixing time lags, makes the control of dissolved oxygen concentration via the manipulation of the feed flow rate difficult and calls for a sophisticated and accurate control system to be used if close control is necessary. Indeed, the control system that was described in Section 3.4.2 proved to be inadequate for maintaining constant dissolved oxygen concentration at 40% of the saturation value. Instead, it oscillated around the set point as it is illustrated in Figure 10 which depicts the trace of the dissolved oxygen concentration for a typical experiment.

In the beginning of the fermentations, when the biomass concentration was small and increasing slowly, there was little demand for oxygen and the dissolved oxygen concentration was essentially equal to the equilibrium (100%) value. As the culture entered the exponential growth phase, the oxygen concentration decreased sharply and eventually reached the set point at which time the control system was activated and glucose feed started. Enough glucose (to about 20 g/l) was supplied.

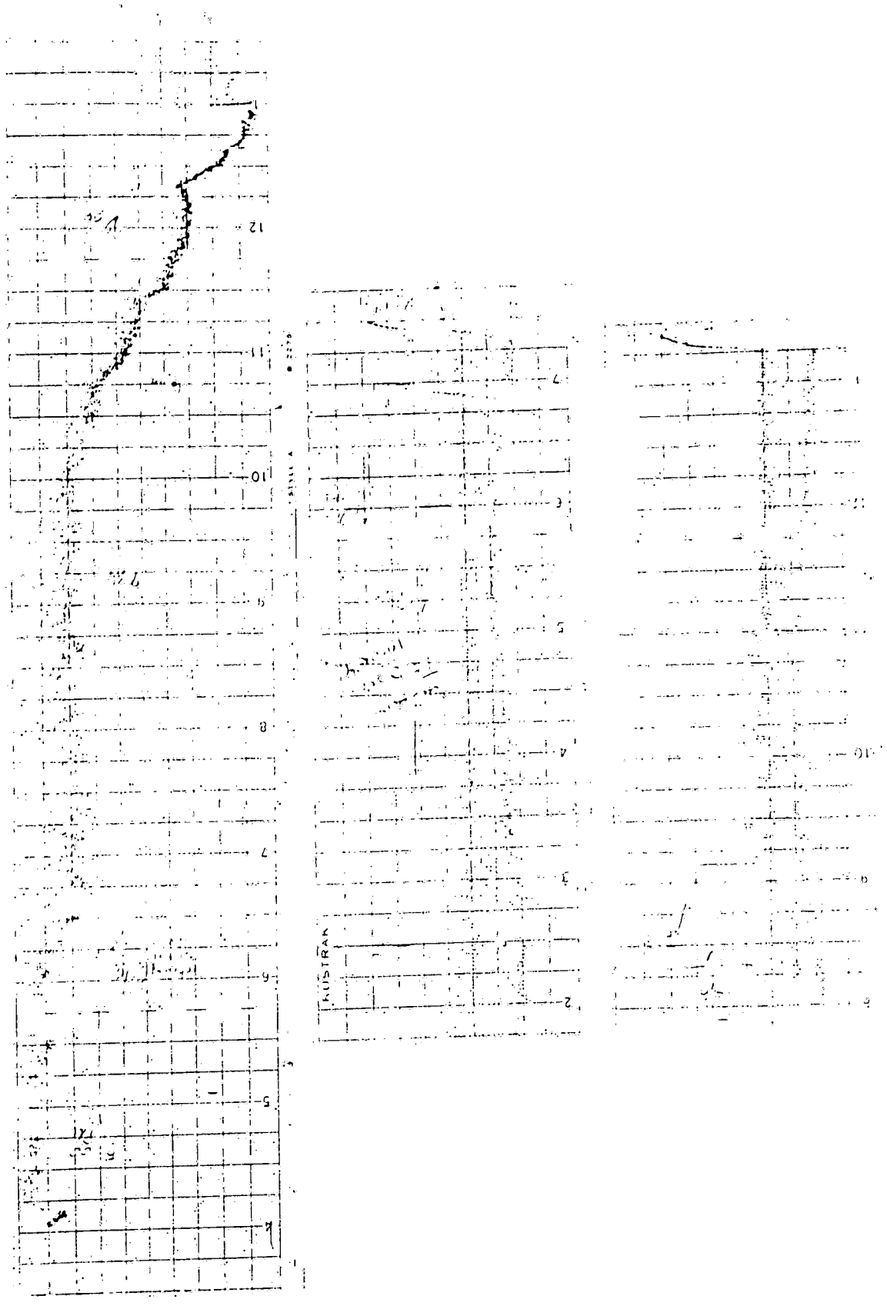


Figure 10: Dissolved Oxygen Trace

before inoculation to support growth to this point. Once the control was operative, the dissolved oxygen concentration oscillated around the set point with frequency and amplitude depending on the pulse length and the speed of the glucose pump. These variables were being adjusted continually during the course of the fermentations to minimize the amplitude of the oscillations. Generally, relatively high flow rates in short pulses gave the best results, since the necessary amount of glucose was added quickly thereby reducing the effect of the time lags. In the bench scale fermenter the dissolved oxygen concentration was maintained between 20% and 50% of saturation with approximately 4 Kg/hr flow of 58% ($^{w}/w$) glucose solution in 0.5 sec pulses. In the deep jet aeration fermenter the oscillations were narrower (25%-43%) for flow rates of approximately 25 Kg/hr of 55% ($^{w}/w$) glucose solution flow in 4.5 sec pulses. The average period of the oscillations was normally about 1.5 minutes.

The oscillations of the dissolved oxygen concentration were the result of the discontinuous addition of glucose caused by the action of the on-off controller. Therefore, the glucose concentration in the broth went through cycles similar to the cycles exhibited by the dissolved oxygen concentration. Although these cycles of the dissolved oxygen and glucose concentrations did not seem to inhibit the growth of the organism, further studies should be done in order to accurately assess their effects.

The experimental growth curves of E coli for the most successful of several runs in the bench scale fermenter and the only run in the

deep jet aeration fermenter are shown in Figure 11. The arrows indicate the times at which the controller was activated and glucose feed started. Besides glucose, ammonia, antifoam and salts were added to the cultures. Regulation of pH was strict; the pH control was trouble free. Unfortunately the salt addition mechanism failed to function properly during the deep jet aeration fermentation being the cause of its short duration. Also, the salts, ammonia and antifoam were added from the steel tanks described in Section 3.4.3.1 and no system was installed to measure accurately the volumes of the additions.

For the bench scale fermentation, 100 ml of 50% ($^{v}/v$) antifoam, 10 ml containing* 9 g $MgSO_4 \cdot 7H_2O$, 12 g K_2HPO_4 and 12 g KH_2PO_4, 250 ml of 18.7% ($^{w}/w$) ammonia and 484 ml of 58% ($^{w}/w$) glucose solution were added to the culture. The final volume of the culture was calculated by adding the volumes of the above additions to the initial volume of the culture. The rate of the volume increase was assumed directly proportional to the known rate of glucose addition (Figure 12). In other words, a uniform feed stream of fixed glucose concentration was assumed as in the simulation. Comparison of the calculated final volume with the measured value revealed a 650 ml discrepancy, the volume of the samples being taken into account. Assuming that the air is sparged at 7.5 lpm, enters at 25 °C and 65% saturated with

*A $MgSO_4 \cdot 7H_2O$ solution (9g in 20 ml of water) was separately from a K_2HPO_4 and KH_2PO_4(24 g total in 50 ml). If these salts were sterilized together, a solid persipitate, complex potassium-magnesium salt, would form.

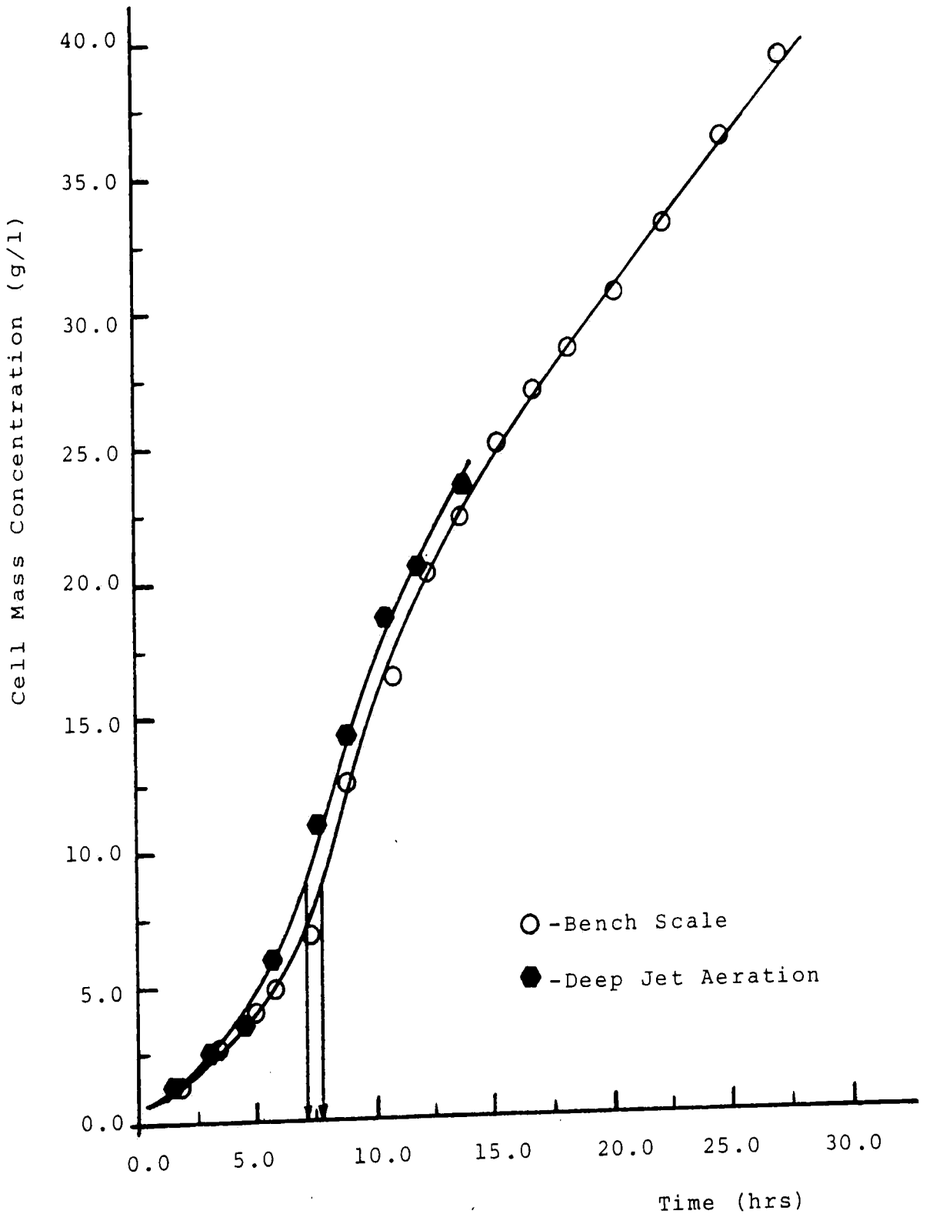


Figure 11: Cell Mass Concentration Versus Time; Experimental Results

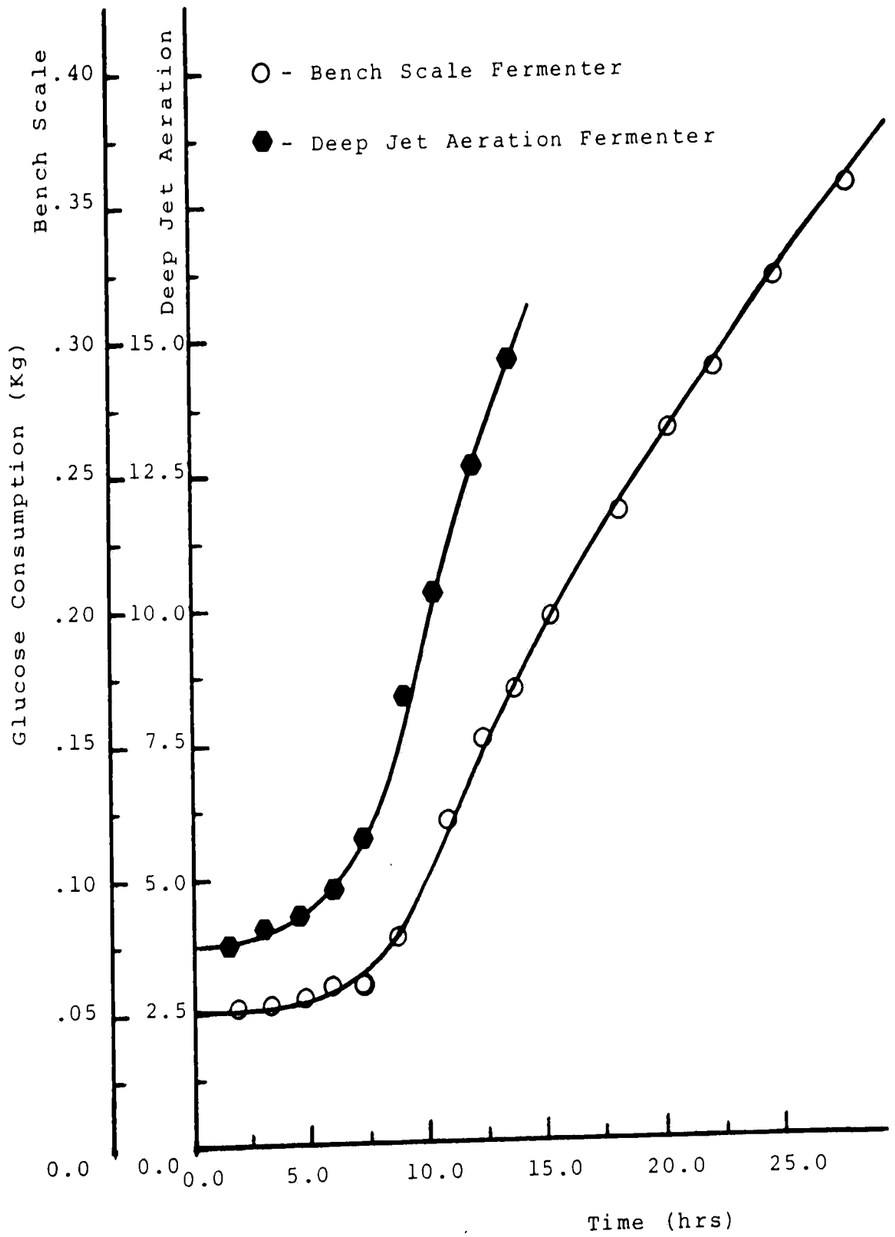


Figure 12: Glucose Consumption Versus Time; Experimental Results.

water, and leaves the fermenter at 35 °C and 95% saturation, a rough calculation gives approximately 400 ml loss due to evaporation during 30 hrs of fermentation. The remaining volume loss can be attributed to ammonia stripping, carbon dioxide emission and error in volume estimations (initial, final, samples and additions).

The measured cell concentrations for each sampling time were corrected to eliminate the effect of the volume loss: The cell concentrations were multiplied by their respective ratios of volumes measured at each sampling time over the calculated volumes at each sampling time. No such correction was performed on the experimental results of the deep jet aeration fermentation due to the lack of accurate volumetric data (errors of up to 25% quite possible) on the additions of salts, base and antifoam and the final volume of the culture. Erroneous results in conjunction with the short duration of the run would prevent meaningfull comparison of the deep jet aeration fermentation with the simulated and bench scale fermentations. Therefore, the following discussion is limited to the corrected data from the bench scale experiment and the results obtained from the parametric study on the model of the process. Also, it should be noted that conclusions drawn from comparisons of the experimental results with those of the simulation require validity of the assumptions made in Theory, Section 2. Particularly, the assumptions of constant yield, maintenance and oxygen transfer coefficients and the validity of Monod's Kinetic expression (Eq. 2.4) should be questioned for the rapidly changing substrate and dissolved oxygen concentrations exhibited during the experiments.

The bench scale fermentation's cell concentration data points, adjusted for the volume loss, and the computer generated growth curves for different Monod constants and oxygen transfer coefficient of 500 hr^{-1} (Runs 2, 3, and 4 in Table 4.1) are shown in Figure 13. The arrows again show the times at which the controller was activated. The widely different maximum growth rate, μ_{max}, values do not influence the growth curve in any dramatic way. All curves tend to produce the same quasi steady state where the specific growth rates are the same, independent of μ_{max} and saturation constants K_s, and equal to the dilution rate of the culture (for the curves in Figure 13 the specific growth rate is equal to 0.0171 hr^{-1} at t = 51 hrs). Since the growth rate is the same for all K_s - μ_{max} combinations, the substrate concentration in the broth (Figure 14) is higher for low μ_{max} and high K_s values; consider Figure 9. It is in the beginning of the fermentation, when there is not severe oxygen demand and the growth is unrestricted (high substrate concentrations, $\mu = \mu_{max}$), that the effect of μ_{max} is pronounced. A low μ_{max} value denotes slower unrestricted growth; therefore the times at which the total oxygen demand reaches a value high enough to cause the dissolved oxygen concentration to decrease below the set point increase with lower μ_{max} values. The experimental growth curve for unrestricted growth, i.e., up to the point when the controller was activated gives a calculated specific growth rate of approximately 0.3 hr^{-1} and the time at which the control was operative is greater than that of the simulated curves (Figure 13) as it could be predicted considering the previous discussion. Also, once the control is operative, the experimental growth curve follows

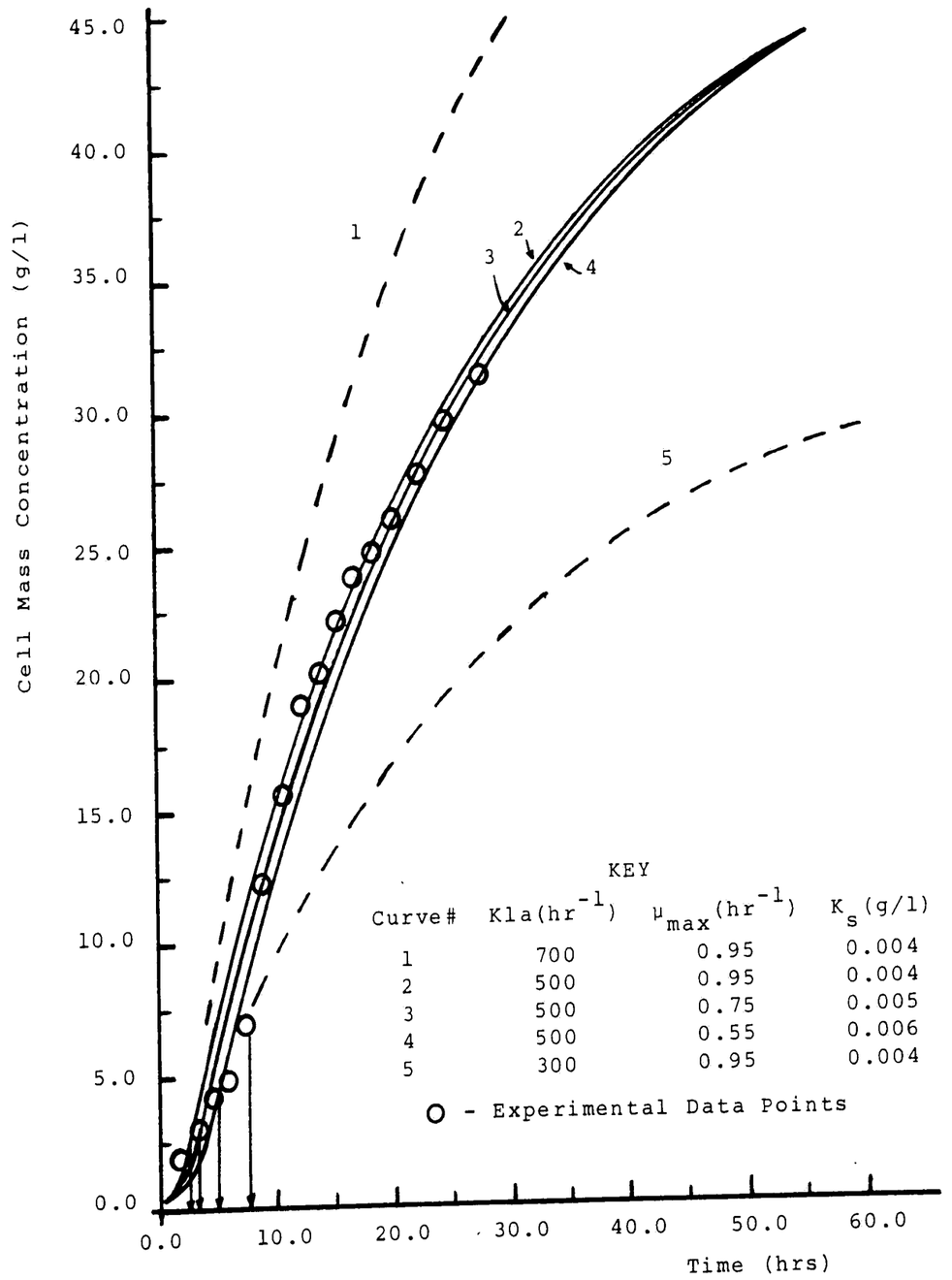


Figure 13: Cell Mass Concentration Versus Time for Different μ_{max} and K_s; Simulation and Bench Scale Experiment.

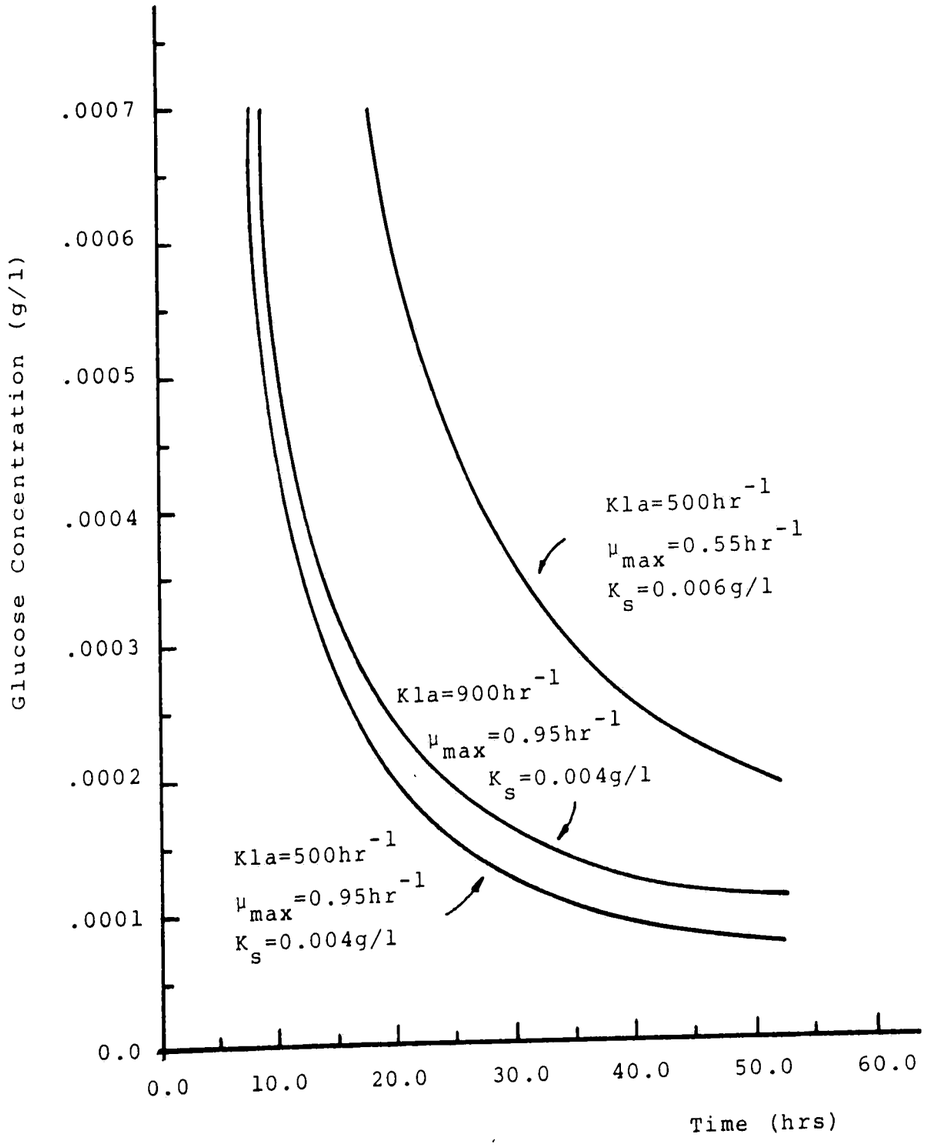


Figure 14: Glucose Concentration Versus Time; Simulation.

the simulated curves, suggesting that the oxygen transfer coefficient is about 500 hr^{-1}, which value is in agreement with literature values for such stirred tank fermenters[24].

The experimental glucose concentration in the broth did not exhibit a diminishing trend as predicted by the simulation (Figure 14). The measured concentrations were all approximately 0.2 g/l, obviously in error considering Figure 9. This error is attributed to the inability of the glucose analyzer to measure accurately very low concentrations. Since there was not enough time between sampling, no elaborate glucose analysis was performed.

The increases of the weights of the total biomasses in the cultures for the simulated and bench scale fermentations are shown in Figure 15. Since there is an almost linear increase in biomass and assuming constant yield and maintenance coefficients, the rate of glucose consumption for cell synthesis is constant and the rate of glucose consumption for cell maintenance increases with time. Therefore, the rate of the total glucose consumption increases and the feed flow rate into the culture increases too causing the total volume versus time plots of Figure 16 to curve upwards. The effect of the glucose maintenance requirement is depicted clearly in Figure 17 where the yield coefficient based on glucose is shown to follow a decreasing pattern as the cell concentration increases. The experimental overall yield coefficient was calculated for the time intervals between inoculation and each sampling. The large deviation of the experimental results

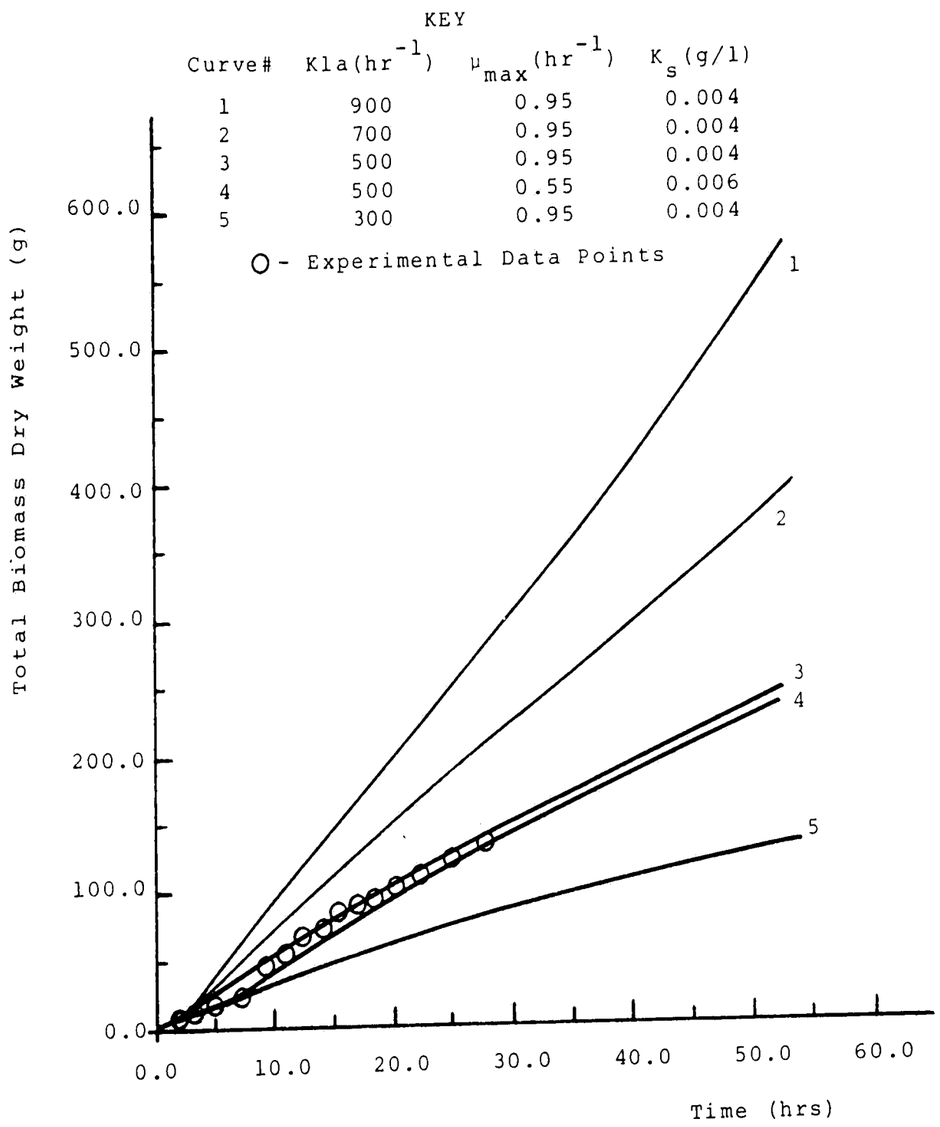


Figure 15: Total Biomass Weight Versus Time; Simulation and Bench Scale Experiment.

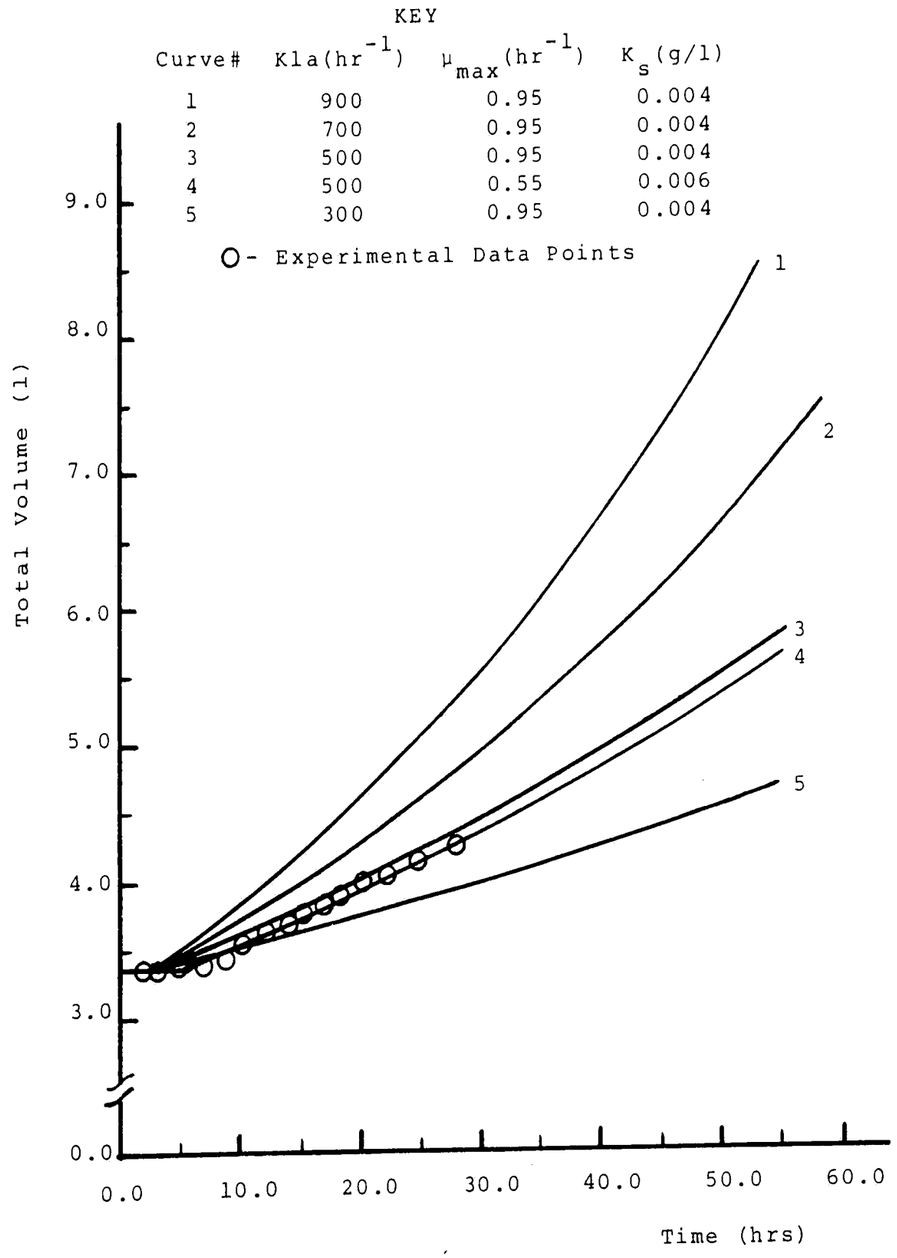


Figure 16: Total Culture Volume Versus Time; Simulation and Bench Scale Experiment.

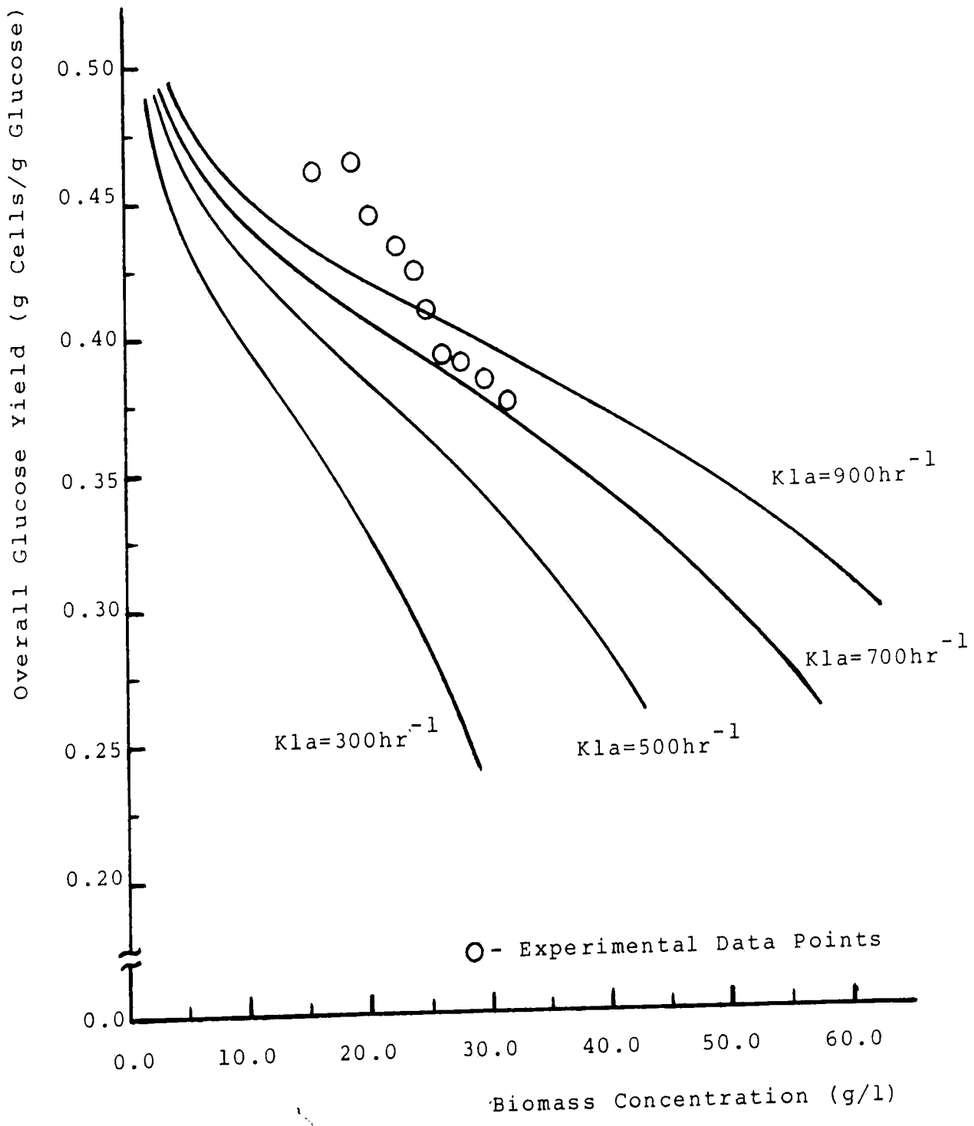


Figure 17: Overall Glucose Yield Versus Cell Mass Concentration. Simulation (K_s=0.004g/l, μ_{max}=0.95 hr^{-1}) and Bench Scale Experiment.

from the yield coefficients of the simulation is attributed to inaccuracies in the volume and glucose concentration measurements. The error is amplified in the beginning of the fermentation because of the small value of the glucose consumed in the denominator of the yield ratio: Δ cell weight/Δ glucose consumed.

Overall, 1350 g of glucose and 46 g of ammonia were consumed for the production of 136 g of E coli placing the ammonia to glucose ratio at 0.13 g NH_3/g Glucose. This ratio, however, is not indicative of the organism's nitrogen consumption because ammonia stripping affected the rate of ammonia addition and the nitrogen dissolved in the culture broth was not measured. It was not an objective of this work to evaluate the rate of ammonia stripping. However, from the series of experiments ran in the bench scale fermenter, it was concluded that ammonia consumption increased with increasing ammonia flow rate, ammonia concentration in the ammonia feed, and aeration and agitation rates, as it could be predicted considering basic mass transfer principles.

Figure 18 presents a rough productivity comparison between repeated batch and repeated fed batch cultivations for E coli production. The unrestricted growth phase of the experiment, before the controller was activated, (Figure 13) is used to approximate batch cultivation and a turnaround time of 2 hours is assumed. Repeated fed batch is considered to be at the quasi steady state because the cell mass concentration versus time curve of Figure 13 does not

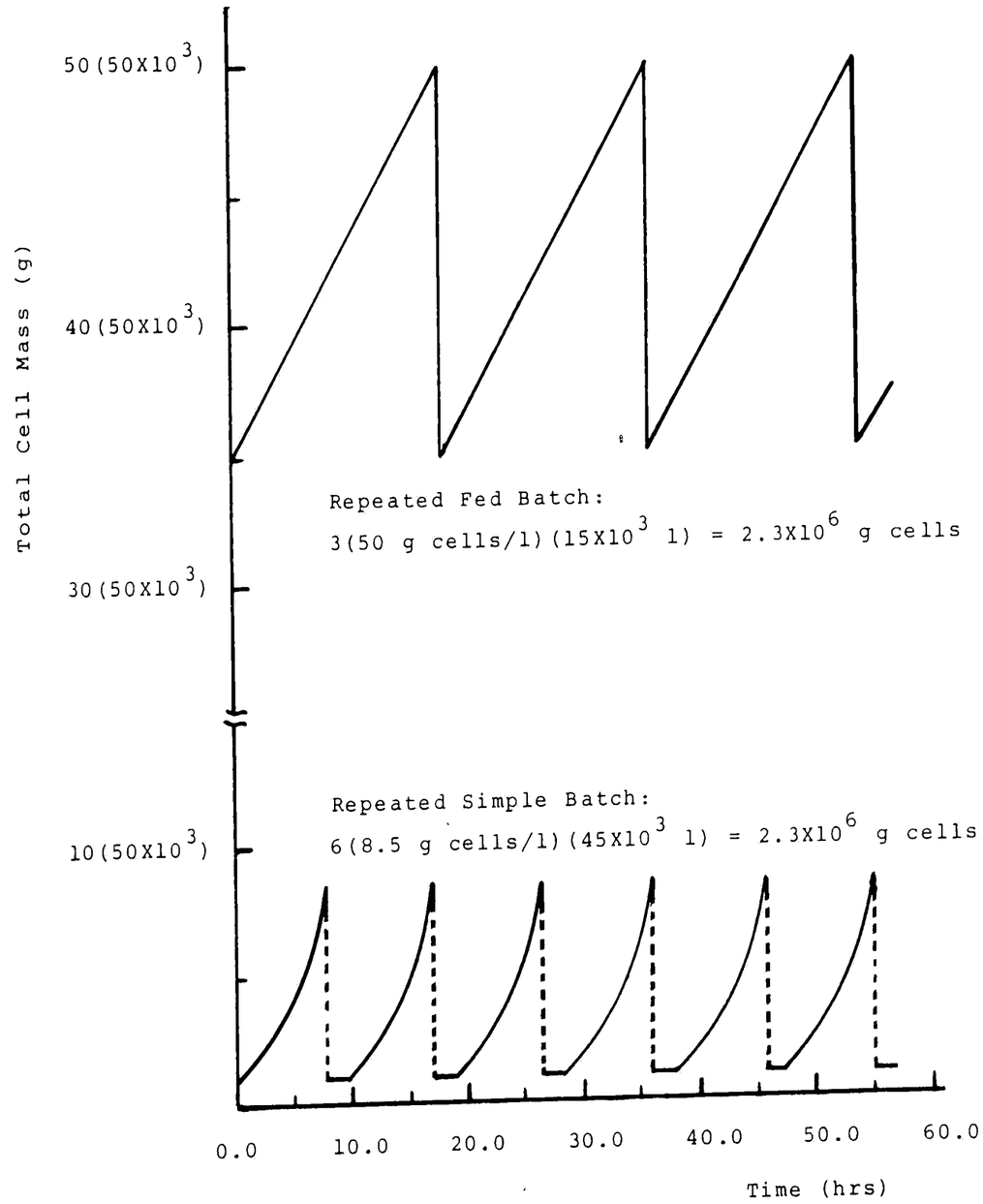


Figure 18: Repeated Fed Batch and Repeated Batch Schedules.

change shape when a portion of the culture's total volume is removed. Also it is assumed that the culture contains 50 g dry cells per l of broth at the quasi steady state. The above cell concentration value was obtained by extrapolating the Kla = 500 hr^{-1} curve in Figure 6. Due to lack of quasi steady state data the culture volume is assumed to increase linearly during the quasi steady state. The rate of volume increase is assumed to be equal to the highest slope of the volume versus time curves of Figure 16 for Kla = 500 hr^{-1} which occurs as the quasi steady state is approached (t = 52 hrs). Since Figure 16 shows that the culture volume increases exponentially the above assumption will yield conservative productivity results for the repeated fed batch process. The capacity of the hypothetical fermenter to be used is taken to be 50,000 l, although this parameter does not influence the results of the comparison and may determine only the turnaround time of the repeated batch fermentation (i.e. the time required to pump the culture out of the fermenter and refill it with fresh medium), which is taken to be 2 hours. Furthermore, 10% of the total volume, which contains 8.5 g cells/l, is assumed to serve as inoculum and account for growth during filling of the fermenter for the repeated batch fermentation. The portion of the total volume that is withdrawn when the maximum capacity is reached during the repeated fed batch cultivation is assumed to be 30% of total volume. The volume portion removed does not affect the productivity of the repeated fed batch cultivation when linear volume increase is assumed at the quasi steady state.

Under the above assumptions, the productivity of repeated fed batch equals that of repeated batch, both giving about 42 Kg dry cells of E coli per hour. However, this is an oversimplified comparison which tends to underestimate the productivity of repeated fed batch by assuming linear volume increase at the quasi steady state; quasi steady state data are essential for accurate assessment of the fed batch cultivation's productivity. Also, since the growth rate is at its lowest value at the quasi steady state (Figure 7), repeated fed batch before the quasi steady state is reached, i.e., a combination of repeated batch - fed batch, may yield the best productivity results depending on the effect of the turnaround time.

In terms of cost effectiveness, repeated fed batch appears to be favorable over repeated batch if one assumes that the cost of pumping, sterilizing agitating and product recovering is directly proportional to the volume of the liquid that has to be processed. The cell concentration of the fermentation product is 50 g/l in the case of repeated fed batch and 8.5 g/l in repeated simple batch (Figure 18). Therefore, the amount of liquid that has to be processed during repeated simple batch cultivation is about 6 times greater than that of repeated fed batch.

6. RECOMMENDATIONS

Although the basic aspects of dissolved oxygen controlled fed batch cultivation have been examined and an experimental method has been established, further work should be done in order to:

a) Improve the accuracy of the experimental results;

b) investigate the performance of the process at the quasi steady state;

c) compare the performance of the deep jet aeration fermenter to that of the conventional stirred tank fermenters;

d) determine the validity of the assumptions embodied in the development of the model of the process and implement the computer simulation;

e) assess the effects of the oscillations of the dissolved oxygen and glucose concentrations and determine whether improved control is necessary.

The major source of error in the experimental results was the inaccuracy of the volume measurements. Therefore, a method for measuring accurately the volume of the culture and of the volume of the additions should be devised. Although the procedures described in this report can be improved by recording carefully the weight of all addition vessels at short and regular time intervals, the use of an integrated feed which will contain all nutrients (glucose salts and trace elements) for balanced additions should be considered. Accurate volumetric data could be taken by recording the flow rate of

the feed. Ammonia or another nitrogen source could be included in the integrated feed if the pH can be thus controlled.

Accurate measurement of the glucose concentration in the culture broth is difficult due to the extremely low values encountered and the high rate of glucose utilization. However, rapid sampling techniques and an analytical method more accurate than the glucose analyzer (e.g. DNS) should be considered.

The organism's nitrogen consumption (nitrogen yield) should also be evaluated, which requires accurate measurement of the amount of ammonia added, evaluation of the ammonia loss due to stripping and measurement of the culture's nitrogen content.

The above suggestions could improve the accuracy and the effectiveness of the experiments which should be designed for long duration to investigate the performance of fed batch cultivation at the quasi steady state. Quasi steady state data are needed in order to determine the productivity of repeated fed batch cultivation. Also, Figures 6 and 13 imply that the oxygen transfer coefficient is the determinant parameter of the maximum biomass concentration attainable at the quasi steady state. Therefore, cell concentration data at the quasi steady state can provide an alternative way of evaluating the oxygen transfer characteristics of the deep jet aeration fermenter when compared to the simulation results and the bench scale stirred tank fermenter.

The computer simulation can be extended to incorporate the measurement and mixing time lags, which can be determined experimentally. Also, better values for the model parameters that characterize the microorganism should be obtained experimentally. Then, any control system can be evaluated and tuned for performance and quasi steady state stability by means of the simulation before it is purchased and used for real fermentations which are quite expensive considering the vapacity of the Vogelbush deep jet aeration fermenter. Furthermore, extensive parametric studies on any model parameter and a search for an optimum productivity schedule can be performed by the use of the computer.

Finally, the effect of the oscillations of the dissolved oxygen and glucose concentrations, that result from the use of pulse on-off control, should be assessed. If they are harmfull (no such indication can be concluded from the experimental results) and must be eliminated, a sophisticated control system should be used. Proportional - integral - derivative control, where the derivative action can help to compensate for the effect of the time lags, is recommended if the signal to the controller is proved to be noise free[11]. Furthermore, galvanic probes such as the one used here, with time constants of about 14 sec[19,25] (Eq. 3.13) are the slowest. Polarographic probes are considerably faster with time constants of about 7 sec[19]. The advantages resulting from the use of a polarographic probe, i.e., shorter measurement time lag, greater stability - less noise, decreased drift during the fermentations and longer life, may well justify their high cost.

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APPENDIX

The following pages present a complete run (program - output) of the computer program used for the simulation.

For this particular run (No 4 in Table 5.1),

K_s = 0.006 g/l

μ_{max} = 0.55 hr^{-1}

Kla = 500 hr^{-1}

```
      PROGRAM ECOLI (INPUT,CUTPUT,TAPE5=INPUT,TAPE6=CUTPUT)
C...
C THIS PROGRAM WAS WRITTEN BY OTHON STACHTIARIS FOR THE BIOTECHNOLOGY
C RESEARCH CENTER AT LEHIGH UNIVERSITY.  IT SIMULATES THE FED BATCH
C GROWTH OF E-COLI UNDER SUBSTRATE LIMITATION INDUCED BY OXYGEN ABSORPTION
C RATE CONTROLLED SUBSTRATE FEEDING.
C THE INTERGRATOR USED TO SOLVE THE SYSTEM OF ODES THAT DESCRIBE THE
C SYSTEM IS THE *LIVERMORE SOLVER FOR ORDINARY DIFFERENTIAL EQUATIONS*,
C AUGUST 13, 1981 VERSION.
C...
C REFERENCE..
C       ALAN C. HINDMARSH, LSODE AND LSODI, TWO NEW INITIAL VALUE
C       ORDINARY DIFFERENTIAL EQUATION SOLVERS,
C       ACM-SIGNUM NEWSLETTER, VOL. 15, NO. 4 (1980), PP. 10-11.
C...
C AUTHOR AND CONTACT...  ALAN C. HINMARSH,
C                        MATHEMATICS AND STATISTICS DIVISION, L-316
C                        LAWRENCE LIVERMORE NATIONAL LABORATORY
C                        LIVERMORE, CA 94550
C...
C...
C THE PARAMETERS IN THE PROGRAM HAVE THE FOLLOWING MEANINGS.
C   MODEL PARAMETERS..
C   RI.. BROTH DENSITY. (GR/LT)
C   KLA.. OXYGEN TRANSFER COEFFICIENT. (1/HR)
C   CEQ.. EQUILIBRIUM OXYGEN CONCENTRATION. (GR/LT)
C   CSP.. SET POINT OXYGEN CONCENTRATION. (GR/LT)
C   KS.. SATURATION CONSTANT IN THE MONOD EXPRESSION. (GR/LT)
C   MMX.. MAXIMUM GROWTH RATE IN THE MONOD EXPRESSION. (1/HR)
C   YG,YO.. GLUCOSE AND OXYGEN YIELDS.
C   MG,MO.. GLUCOSE AND OXYGEN MAINTENANCE COEFFICIENTS. (1/HR)
C   YC.. GLUCOSE CONCENTRATION IN THE FEED STREAM. (GR/LT)
C   FO,FF,E,KC,VX,CTR.. CONTROL SCHEME PARAMETERS.
C ALL OTHER PARAMETERS ARE USED BY LSODE AND PRTINT
C...
C...
C...
      COMMON/T/TIME/Y/S,V,X,C/F/DSDT,DVDT,DXDT,DCDT
     +/PARM/IP,M,N,NPAGE,NLINE,RF,RI,KLA,CEQ,CSP,CTR,
     +KS,MMX,YG,YO,YC,MG,MO,FO,FF,E,KC,VX
      EXTERNAL FEX,DERV,PRINT
      DIMENSION ATOL(4),RWORK(74),IWORK(24)
      REAL KS,MMX,KLA,MG,MO,KC
      DIMENSION Y(4)
      IP=0
      M=1
      N=1
      NPAGE=1
      NLINE=0
C...
C     TO SET THE VALUES OF THE MODEL CONSTANTS
C...
      YG=0.5
      MG=0.056
      YO=0.875
      MO=0.032
      KS=0.0060
```

```
      MMX=0.55
      KLA=500.
      CEQ=0.0074
      CSP=0.00296
      CTR=0.0015
      YC=0.36
      FO=0.00
      FF=4000.0
      KC=8500.0
      PI=1050.0
C...
C     TO INITIALIZE THE DEPENDENT VARIABLES
C...
      Y(1)=8.5
      Y(2)=3.35
      Y(3)=0.3
      Y(4)=CEQ
C...
C     TO SET ALL PARAMETERS NEEDED FOR THE INTEGRATOR.
C...
      IOPT=1
      DO 10 L=5,10
      RWORK(L)=0.0
      IWORK(L)=0
   10 CONTINUE
      IWORK(6)=1000
      NEQ=4
      T=0.0
      TOUT=0.25
      ITOL=2
      RTOL=1.0E-3
      ATOL(1)=1.0E-6
      ATOL(2)=1.0E-3
      ATOL(3)=1.0E-4
      ATOL(4)=1.0E-7
      ITASK=1
      ISTATE=1
      LRW=74
      LIW=24
      MF=22
C...
C     TO SET THE PRINTING INTERVAL, TO CALL THE INTERGRATOR AND TO
C     PRINT USEFULL STATISTICAL INFORMATION.
C...
      DO 40 IOUT=1,100
C...
C     LSODE IS NOT PROVIDED IN THIS PRINTOUT
C...
      CALL LSODE(FEX,NEQ,Y,T,TOUT,ITOL,RTOL,ATOL,ITASK,ISTATE,
     +           IOPT,RWORK,LRW,IWORK,LIW,JEX,MF)
      NI=5
      NO=6
C...
C     NOTE THAT PRINT AND DERV NEED NOT BE SOUBROUTINES
C...
      CALL PRINT(NI,NO)
      IF(ISTATE.LT.0)GOTO80
```

```
   40 TOUT=TOUT*1.055
      WRITE(NO,60)IWORK(11),IWORK(12),IWORK(13)
   60 FORMAT(///,25X,
     +         12HNO. STEPS = ,I4,11H  NO. F-S =,I4,11H  NO. J-S =,I4)
      STOP
   80 WRITE(NO,90)ISTATE
   90 FORMAT(///,22H ERROR HALT.. ISTATE =,I3)
      STOP
      END
```

```
      SUBROUTINE FEX(NEQ,T,Y,YDOT)
      DIMENSION Y(4),YDOT(4)
      COMMON/T/TIME/Y/S,V,X,C/F/DSDT,DVDT,DXDT,DCDT
     +/PARM/IP,M,N,NPAGE,NLINE,PF,PI,KLA,CEQ,CSP,CTR,
     +KS,MMX,YG,YO,YC,MG,MO,FO,FF,E,KC,VX
      REAL KS,MMX,KLA,MG,MO,KC
C...
C     TO INTRFACE FEX WITH DERV AND TO CALL DERV
C...
      S=Y(1)
      V=Y(2)
      X=Y(3)
      C=Y(4)
      TIME=T
      CALL DERV
      YDOT(1)=DSDT
      YDOT(2)=DVDT
      YDOT(3)=DXDT
      YDOT(4)=DCDT
      RETURN
      END
```

```
      SUBROUTINE DERV
      COMMON/T/TIME/Y/S,V,X,C/F/DSDT,DVDT,DXDT,DCDT
     +/PARM/IP,M,N,NPAGE,NLINE,RF,RI,KLA,CEQ,CSP,CTR,
     +KS,MMX,YG,YO,YC,MG,MO,FO,FF,E,KC,VX
      REAL KS,MMX,KLA,MG,MO,KC
C...
C     TO PREVENT SUBSTRATE INTRODUCTION BEFORE THE DISSOLVED
C     OXYGEN CONCENTRATION DECREASES BELOW THE SET POINT
C     FOR THE FIRST TIME
C...
      IF(M.LT.2)GOTO1
C...
C     THE FOLLOWING STATEMENTS SIMULATE THE ACTION OF THE
C     PROPORTIONAL CONTROLLER.  THE FLOW INTO THE FERMENTOR STARTS WHEN
C     THE D.O. CONCENTRATION  EXEEDS THE SET POINT D.O. CONCENTRATION.
C...
C      CONTROLLER EQUATIONS
C...
      E=(CSP-C)
      VX=-KC*E
      IF(VX.LT.0.)VX=0.0
      IF(VX.GT.1.)VX=1.0
C...
C      VALVE EQUATIONS.  HERE CV AND SQRT(DP/SP.GR.)
C      IS INCORPORATED INTO ONE FIGURE.
C...
      F=FF*VX
      GOTO4
    1 F=FO
      IF(C.LE.CTR)GOTO3
      GOTO4
    3 M=M+1
      IF(M.GT.20)M=20
    4 CONTINUE
C...
C     SYSTEM'S MODEL ODES
C...
      DSDT=F*(YC-S/RI)/V-(MMX*S/(KS+S))*X/YG-MG*X
      DVDT=F/RI
      DXDT=(MMX*S/(KS+S)-F/(RI*V))*X
      DCDT=KLA*(CEQ-C)-(MMX*S/(KS+S))*X/YO-F*C/(V*RI)-MO*X
      RETURN
      END
```

```
      SUBROUTINE PRINT(NI,NO)
      COMMON/T/TIME/Y/S,V,X,C/F/DSDT,DVDT,DXDT,DCDT
     +/PARM/IP,M,N,NPAGE,NLINE,PF,PI,KLA,CEQ,CSP,CTP,
     +KS,MMX,YG,YO,YC,MG,MO,FO,FF,F,KC,VX
      REAL KS,MMX,KLA,MG,MO,KC
      IF(NLINE.GT.0)GOTO300
      WRITE(NO,400)
  400 FORMAT(1H1)
      WRITE(NO,200)NPAGE
  200 FORMAT(//,65X,5HPAGE ,I3,///,13X,
     +4HTIME,5X,9HSUBSTRATE,4X,5HTOTAL,5X,4HCELL,
     +6X,9HDISSOLVED,1X,6HGROWTH,1X,7HGLUCOSE,/,
     +12X,7HELAPSED,1X,13HCONCENTRATION,1X,6HVOLUME,1X,
     + 13HCONCENTRATION,3X,6HOXYGEN,3X,4HRATE,3X,5HYIELD,/,
     +13X,5H(HRS),6X,7H(GR/LT),4X,4H(LT),5X,7H(GR/LT),
     +5X,7H(GR/LT),2X,6H(1/HR),1X,7H(GR/GR),/)
      NLINE=NLINE+8
  300 RG=MMX*S/(S+KS)
      IF(V.LE.3.352)GOTO 500
      OY=(V*X-3.35*0.3)/((V-3.35)*1160.0*0.36-S*V+3.35*8.5)
  500 WRITE(NO,100)(TIME,S,V,X,C,RG,OY)
  100 FORMAT(13X,F5.2,4X,F8.5,4X,F6.3,5X,F6.3,4X,F8.6,2X,F6.4,2X,F6.3)
   10 N=N+1
      NLINE=NLINE+1
      IF(NLINE.LT.55)RETURN
      NPAGE=NPAGE+1
      NLINE=0
  600 CONTINUE
      RETURN
      END
```

TIME ELAPSED (HRS)	SUBSTRATE CONCENTRATION (GR/LT)	TOTAL VOLUME (LT)	CELL CONCENTRATION (GR/LT)	DISSOLVED OXYGEN (GR/LT)	GROWTH RATE (1/HR)	GLUCOSE YIELD (GR/GR)
.27	8.39914	3.350	.348	.005941	.5496	-I
.27	8.39914	3.350	.348	.005941	.5496	-I
.52	8.28702	3.350	.401	.005871	.5496	-I
.52	8.28702	3.350	.401	.005871	.5496	-I
.52	8.28702	3.350	.401	.005871	.5496	-I
.52	8.28702	3.350	.401	.005871	.5496	-I
.52	8.28702	3.350	.401	.005871	.5496	-I
.52	8.28702	3.350	.401	.005871	.5496	-I
.52	8.28702	3.350	.401	.005871	.5496	-I
.52	8.28702	3.350	.401	.005871	.5496	-I
.52	8.28702	3.350	.401	.005871	.5496	-I
.52	8.28702	3.350	.401	.005871	.5496	-I
.52	8.28702	3.350	.401	.005871	.5496	-I
.77	8.16257	3.350	.461	.005793	.5496	-I
.77	8.16257	3.350	.461	.005793	.5496	-I
.77	8.16257	3.350	.461	.005793	.5496	-I
.77	8.16257	3.350	.461	.005793	.5496	-I
.77	8.16257	3.350	.461	.005793	.5496	-I
.77	8.16257	3.350	.461	.005793	.5496	-I
1.03	8.01795	3.350	.529	.005702	.5496	-I
1.03	8.01795	3.350	.529	.005702	.5496	-I
1.03	8.01795	3.350	.529	.005702	.5496	-I
1.03	8.01795	3.350	.529	.005702	.5496	-I
1.03	8.01795	3.350	.529	.005702	.5496	-I
1.39	7.77284	3.350	.646	.005548	.5496	-I
1.39	7.77284	3.350	.646	.005548	.5496	-I
1.39	7.77284	3.350	.646	.005548	.5496	-I
1.39	7.77284	3.350	.646	.005548	.5496	-I
1.39	7.77284	3.350	.646	.005548	.5496	-I
1.39	7.77284	3.350	.646	.005548	.5496	-I
1.75	7.47015	3.350	.790	.005358	.5496	-I
1.75	7.47015	3.350	.790	.005358	.5496	-I
1.75	7.47015	3.350	.790	.005358	.5496	-I
1.75	7.47015	3.350	.790	.005358	.5496	-I
2.11	7.10395	3.350	.964	.005129	.5495	-I
2.11	7.10395	3.350	.964	.005129	.5495	-I
2.11	7.10395	3.350	.964	.005129	.5495	-I
2.48	6.65754	3.350	1.177	.005849	.5495	-I
2.48	6.65754	3.350	1.177	.005849	.5495	-I
2.48	6.65754	3.350	1.177	.005849	.5495	-I
2.98	5.86660	3.350	1.553	.005353	.5494	-I
2.98	5.86660	3.350	1.553	.005353	.5494	-I
2.98	5.86660	3.350	1.553	.005353	.5494	-I
2.98	5.86660	3.350	1.553	.005353	.5494	-I

TIME ELAPSED (HRS)	SUBSTRATE CONCENTRATION (GR/LT)	TOTAL VOLUME (LT)	CELL CONCENTRATION (GR/LT)	DISSOLVED OXYGEN (GR/LT)	GROWTH RATE (1/HR)	GLUCOSE YIELD (GR/GR)
3.48	4.82592	3.350	2.048	.004700	.5493	-I
3.48	4.82592	3.350	2.048	.004700	.5493	-I
3.48	4.82592	3.350	2.048	.004700	.5493	-I
3.98	3.47280	3.350	2.692	.003853	.5491	-I
3.98	3.47280	3.350	2.692	.003853	.5491	-I
4.40	2.01597	3.350	3.385	.002943	.5484	-I
4.40	2.01597	3.350	3.385	.002943	.5484	-I
4.62	1.05645	3.350	3.841	.002358	.5469	-I
4.76	.43362	3.350	4.137	.002009	.5425	-I
5.03	.01460	3.356	4.650	.002951	.3898	.471
5.32	.01036	3.366	5.158	.002951	.3483	.465
5.63	.00793	3.377	5.698	.002951	.3130	.460
5.90	.00656	3.386	6.162	.002961	.2872	.456
6.29	.00523	3.400	6.831	.002961	.2562	.450
6.72	.00429	3.415	7.551	.002951	.2292	.445
7.09	.00372	3.428	8.149	.002951	.2104	.441
7.38	.00336	3.438	8.622	.002951	.1972	.439
7.96	.00280	3.459	9.552	.002951	.1751	.433
8.26	.00260	3.469	10.009	.002951	.1661	.431
8.84	.00225	3.490	10.909	.002951	.1500	.426
9.44	.00198	3.512	11.816	.002951	.1366	.422
10.04	.00176	3.534	12.703	.002961	.1247	.417
10.54	.00162	3.552	13.424	.002951	.1167	.414
11.04	.00149	3.570	14.131	.002951	.1097	.411
11.54	.00138	3.588	14.825	.002951	.1027	.408
12.04	.00129	3.607	15.505	.002961	.0976	.405
12.54	.00120	3.625	16.173	.002951	.0920	.402
13.54	.00107	3.663	17.471	.002951	.0832	.396
14.37	.00098	3.694	18.510	.002951	.0769	.391
15.20	.00090	3.726	19.515	.002961	.0719	.387
16.03	.00083	3.758	20.490	.002951	.0669	.382
17.29	.00074	3.807	21.917	.002951	.0606	.376
17.29	.00074	3.807	21.917	.002951	.0606	.376
18.55	.00067	3.856	23.277	.002951	.0554	.370
19.81	.00062	3.907	24.569	.002951	.0513	.364
21.28	.00056	3.966	26.001	.002951	.0467	.357
21.28	.00056	3.966	26.001	.002951	.0467	.357
22.75	.00051	4.026	27.359	.002951	.0429	.351
24.22	.00046	4.088	28.637	.002951	.0395	.345
25.69	.00044	4.150	29.845	.002951	.0374	.339
26.55	.00042	4.187	30.521	.002951	.0356	.335
28.92	.00037	4.290	32.266	.002951	.0322	.326
30.92	.00034	4.379	33.629	.002961	.0297	.319
32.91	.00032	4.470	34.882	.002951	.0278	.312
32.91	.00032	4.470	34.882	.002951	.0278	.312
36.27	.00028	4.626	36.787	.002951	.0248	.301
39.62	.00026	4.788	38.475	.002952	.0225	.291

TIME ELAPSED (HRS)	SUBSTRATE CONCENTRATION (GR/LT)	TOTAL VOLUME (LT)	CELL CONCENTRATION (GR/LT)	DISSOLVED OXYGEN (GR/LT)	GROWTH RATE (1/HR)	GLUCOSE YIELD (GR/GR)
39.62	.00026	4.788	38.475	.002952	.0225	.291
42.97	.00023	4.956	39.957	.002952	.0204	.282
42.97	.00023	4.956	39.957	.002952	.0204	.282
46.32	.00022	5.130	41.252	.002952	.0191	.273
51.57	.00019	5.414	42.971	.002952	.0172	.260
51.57	.00019	5.414	42.971	.002952	.0172	.260

NO. STEPS = 469 NO. F-S =1532 NO. J-S = 173